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**Towards understanding latrophilin signalling in
*C.elegans***

By

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Abstract

Latrophilin (*LAT-1*) is a G-protein-coupled receptor (GPCR), and mediates Black widow spider venom toxicity (Mee et al., 2004). A deletion in the *lat-1(ok1465)* gene (allele *ok1465*) in *C. elegans* causes 97% of worms to die during embryogenesis or early larval stages (Adenle, 2008). Thus latrophilin has an essential function in development, but it is not known how latrophilin signalling causes biological effects. The aim of this study is to identify genes that mitigate the effect of the *lat-1(ok1465)* deletion allele on offspring lethality, with a view to identifying the pathways involved in latrophilin signalling that mediate developmental lethality.

Brood size was determined for N2 worms (295 ± 36 ; mean \pm standard deviation), whereas *lat-1(ok1465)* worms produced only 4 ± 4 adult offspring ($P < 0.05$). It was decided to undertake mutagenesis of the *lat-1(ok1465)* worms, but this requires bleaching of adult populations, to yield synchronously growing populations. While ~0% of wild type L1 died after bleaching, *lat-1(ok1465)* worms showed ~97% lethality. The recovery of worms after bleaching of *lat-1(ok1465)* worms was 0.07 offspring per input adult worm. The mutagenesis procedure involves three generations of worms with bleaching at each stage, and so around 6 million *lat-1(ok1465)* adult worms at P_0 are required to get 20,000 F_2 L4 worms. Liquid culture is required to grow this large number of worms, and worm growth in liquid media was optimised. A strategy for screening *lat-1(ok1465)* worms was devised; screening the brood size of 2×10^4 individual worms is prohibitively time-consuming, and so worms were screened in plates containing 20 *lat-1(ok1465)* worms. 20 *lat-1(ok1465)* worms would be expected to yield around 80 offspring, and experimental testing showed that there were 74 ± 14 worms per plate. To date, screening of 10000 F_2 from mutagenised *lat-1(ok1465)* worms has been performed. Thirty independent plates were shown to have worms with the multi-vulva

phenotype, a mutation rate of 0.6 per 1000 mutagenised genomes. The screen yielded two plates with >100 offspring, and these plates yielded two mutant lines whose brood size was statistically significantly increased ($P < 0.05$) compared with the *lat-1(ok1465)* brood size.

This work shows that mutagenesis of *lat-1(ok1465)* worms was successful, and that the screening procedure can be used to yield *lat-1(ok1465)* mutants with increased brood size. Future work will involve mapping and identifying the role of these mutant genes that confer increased brood size to *lat-1(ok1465)* worms.

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Finally, a big thank to my parents and my family for constant support, endless patience and encouragement when I required them.

DEDICATION

THIS THESIS IS DEDICATED TO:

**MY FATHER,
AWAD**

**MY MOTHER,
GAZWA**

**MY WIFE,
AFAF**

**MY DAUGHTER,
RAMA,**

**FINALLY, THIS THESIS IS DEDICATED TO THE MEMORIES OF MY
BROTHERS AND SISTERS TO COMPLETE MY STUDYING**

Abbreviations:

Bp	Base pair
LB	Luria-Bertani
<i>C. elegans</i>	<i>Caenorhabditis elegans</i>
DNA	Deoxyribonucleic acid
E.coli	Escherichia coli
L1-L4	C.elegans larval stages
NGM	Nematode growth medium
O.D	Optical density
PCR	Polymerase chain reaction
SNP	Single nucleotide polymorphism
TEMED	N,N,N',N' - Tetramethylethylenediamine
EMS	Ethyl methane sulfonate

Table of contents

TOWARDS UNDERSTANDING LATROPHILIN SIGNALLING IN <i>C.ELEGANS</i>	1
1. INTRODUCTION	11
SECTION 1.1 <i>CAENORHABDITIS ELEGANS</i>	11
Section 1.1.1 <i>Caenorhabditis elegans</i> as a model system	11
Section 1.1.2 Laboratory culture	11
Section 1.1.3 Sex determination in <i>C. elegans</i>	12
Section 1.1.4 Anatomy	13
Section 1.1.5 Life cycle	15
Section 1.1.6 Benefits of selecting <i>C. elegans</i> for studying toxicity	18
Section 1.2.1 Latrophilin/ CIRL (for Ca^{2+} -independent receptor of LTX)	20
Section 1.2.4 Lat-1(ok1465) (ok1465) worms	27
SECTION 1.3 MUTAGENESIS	29
Section 1.3.1 Types of mutagenesis	Error! Bookmark not defined.
Section 1.3.2 Choice of Mutagen	29
Section 1.3.3 Application of Mutagens	30
SECTION 1.4 SUMMARY OF STUDY	35
2. MATERIALS AND METHODS	36
2.1 MATERIALS	36
Table 2.1.1 Nematode Growth Medium (NGM) agar	36
Table 2.1.2 Potassium Phosphate Buffer (pH 6.0)	37
Table 2.1.3 LB Broth –	37
Table 2.1.4 LB Agar	37
Table 2.1.5 K-Medium	37
Table 2.1.6 Egg Isolation Solution	37
Table 2.1.7 Freezing down Solution	38
Table 2.1.8 Lysis Buffer	38
Table 2.1.9 12% TBE-PAGE Gel	39
Table 2.1.10 TBE	39
Table 2.1.11 PCR Master Mix	39
Table 2.1.12 Loading Buffer	40
2.1.13 Primers	40
Table 2.1.15 S. Basal	41
TABLE 2.1.16 S MEDIUM	41
Table 2.1.17 EMS	41
2. METHODS	42
SECTION 2.2.1 E.COLI CULTURES	42
SECTION 2.2.2 GEL ELECTROPHORESIS	42
SECTION 2.2.3 <i>C.ELEGANS</i> METHODS	43
Section 2.2.3.1 Growth and Maintenance of <i>C.elegans</i>	43
Section 2.2.3.2 Worm Harvesting	44
Section 2.2.3.3 Preparation of synchronous cultures	44
Section 2.2.3.4 Freezing worm stocks	45
Section 2.2.3.5 Transferring worms grown on NGM plates (Culturing of <i>C.elegans</i>)	46
SECTION 2.2.4 ASSAYS FOR DEVELOPMENTAL AND BEHAVIORAL PHENOTYPES IN <i>C.ELEGANS</i>	47
Section 2.2.4.1 Measurement of brood size for N2	47
Section 2.2.4.2 Microscopy of <i>c.elegans</i>	48
SECTION 2.2.5 GROWTH OF <i>C. ELEGANS</i> IN LIQUID MEDIUM	48
Section 2.2.5.1 Liquid medium	49
Section 2.2.5.2 Harvesting worms to isolate embryos for starting a synchronized culture.	50
SECTION 2.2.6 MUTAGENESIS	52
Section 2.2.6.1 EMS method	52
Section 2.2.6.2 Culturing Lat-1(ok1465) worms for mutagenesis	53

Section 2.2.6.3 Screening F2 mutants.....	57
SECTION 2.2.7 POLYMERASE CHAIN REACTION (PCR).....	57
3. RESULTS.....	59
SECTION 3.1 BROOD SIZE ANALYSIS.....	59
Section 3.1.1 Establish the brood size of <i>lat-1(ok1465)</i> worms and Hawaiian background.....	59
SECTION 3.2 YIELD OF WORMS AFTER BLEACHING.....	60
SECTION 3.3 THE NUMBER OF <i>LAT-1(OK1465)</i> FOR MUTAGENESIS.....	61
SECTION 3.4 GROWING WORMS IN LIQUID CULTURE.....	62
Section 3.4.1 Growth of N2 worms in liquid culture.....	63
Section 3.4.2 Growth N2 worms at 1.7 and 1.5 of O.D. 600nm.....	65
Section 3.4.3 use of a large inoculum of N2 worms.....	65
Section 3.4.4 growth of <i>lat-1(ok1465)</i> worms.....	66
Section 3.4.5 Growing large number of <i>Lat-1(ok1465)</i> worm.....	68
SECTION 3.5 MUTAGENESIS.....	69
Section 3.5.1 Size of the mutagenesis screen.....	69
Section 3.5.2 The number of F2 Animals Screening on a single plate.....	70
Section 3.5.3 mutagenesis <i>lat-1(ok1465)</i> worms.....	72
Section 3.5.4 The screening plates for mutant with an increase brood size.....	73
Section 3.5.4.1 Brood size of two candidate mutant lines.....	74
SECTION 3.6 POLYMERASE CHAIN REACTION (PCR).....	76
4. DISCUSSION.....	78
SECTION 4.1 LIQUID CULTURE.....	78
SECTION 4.2 THE <i>LAT-1(OK1465)</i> MUTATION.....	79
SECTION 4.3 MUTAGENESIS <i>LAT-1(OK1465)</i> WORM.....	79
SECTION 4.4 DIFFICULTY OF DEALING WITH <i>LAT-1</i>	80
Section 4.4.1 Screening of <i>lat-1(ok1465)</i> worms.....	80
Section 4.4.2 Culturing <i>lat-1(ok1465)</i> worms for mutagenesis.....	81
SECTION 4.5 SCREENING PLATES FOR <i>LAT-1(OK1465)</i> MUTANT WITH AN INCREASE BROOD SIZE.....	82
Section 4.5.1 Characterization of <i>lat-1(ok1465)</i> mutant with an increase brood size.....	83
SECTION 4.7 A GENETIC MAPPING SYSTEM IN <i>CAENORHABDITIS ELEGANS</i>	84
5. CONCLUSION AND FUTURE WORK.....	86
6. REFERENCES.....	87

List of figures

Figure 1.1: Photomicrograph showing major anatomical feature of XO males (above) and XX Hermaphrodite.....	14
Figure 1.2: This figure shows embryonic stage of development at 22 °C	16
Figure 1.3: Life cycle of <i>C. elegans</i> at 22°C.....	18
Figure 1.4: This figure shows the structural domains of latrophilin / CIRL1-3 and the activation of LHP receptor.....	23.
Figure 1.5: This figure shows the Mechanisms of alpha-latrotoxin action.....	25
Figure 1.7: shows diagram of the ok1465 gene.....	29
Figure 1.8: how the mutagen affects every single worm.....	33
Figure 1.9: This figure shows the Mechanism of mutagenesis by EMS.....	35
Figure 2.2.1: The mutagenesis method.....	56
Figure 3.1: The growth of N2 worms at different bacterial concentration.....	64
Figure 3.2: Growth of N2 worms in liquid culture.....	66
Figure 3.3: <u>U</u> se of a large of N2 worms in liquid culture.....	67
Figure 3.4: The growth of <i>Lat-1(ok1465)</i> worms at O.D. of 1.6 at 600 nm.....	68
Figure 3.5: Multi-vulva phenotype.....	74
Figure 3.6: Testing the two mutant worms.....	76
Figure 3.7: Acrylamide gel showing the specificity of <i>lat-1(ok1465)</i> gene primers.....	78

List of Tables

Table 1.1: This table shows the growth and lifespan of the <i>C. elegans</i> at 16, 20 and 25 °C.....	17
Table 2.1.1 Nematode Growth Medium (NGM) agar.....	37
Table 2.1.2 Potassium Phosphate Buffer (pH 6.0).....	38
Table 2.1.3 LB Broth.....	38
Table 2.1.4 LB Agar.....	38
Table 2.1.5 K-Medium.....	38
Table 2.1.6 Egg Isolation Solution.....	38
Table 2.1.7 Freezing down Solution.....	39
Table 2.1.8 Lysis Buffer.....	39
Table 2.1.9 12% TBE-PAGE Gel.....	40
Table 2.1.10 TBE.....	40
Table 2.1.11 PCR Master Mix.....	40
Table 2.1.12 Loading Buffer.....	41
Table 2.1.13 M9 Buffer.....	41
Table 2.1.14 S. Basal.....	42
Table 2.1.15S Medium.....	42
Table 2.1.16EMS.....	42
Table 3.1 Recovery of <i>N2</i> worms after bleaching.....	61
Table 3.2 Recovery of <i>lat-1(ok1465)</i> worms after bleaching a particular number of <i>Lat-1(ok1465)</i> worms.....	62
Table 3.3 The number of <i>Lat-1(ok1465)</i> for mutagenesis.....	63
Table 3.4: The number of genomes required for screening.....	71
Table 3.5: Screening the number of worms per plate.....	72

1. Introduction

Section 1.1 *Caenorhabditis elegans*

Section 1.1.1 *Caenorhabditis elegans* as a model system

Caenorhabditis elegans (*C. elegans*) are free-living soil nematodes, which are small, multicellular, non-parasitic, invertebrate, eukaryotes studied and popularised by Brenner in 1965 (Brenner, 1974 and Schwarz et al., 2006). *C. elegans* are now widely used in a variety of types of biological research. The reasons for using *C. elegans* are that they have a small size (between ~1mm and 35mm in a large adult), large brood size and short development time (3.5 days at 25° C from egg to adult). *C. elegans* are capable of reproducing large numbers of offspring (>300) by self fertilisation for a hermaphrodite or by mating with male. *C. elegans* is useful for genetic studies because they have a small genome of approximately 100.2 Mb (100,269,912 bases). Self fertilisation and a short life cycle are important for isolation and maintenance of mutant strains, and allow homozygous worms to breed quickly without further mutation. *C. elegans* are a useful tool because they share 40% of their genes (protein sequence) with humans. Furthermore, the body of the worm is transparent and the anatomical features and cell structure have been examined in depth. These advantages mean that *C. elegans* are a valuable tool in many areas of biology such as genetics, cell death, and neuroscience, aging, signalling pathways, animal development and behaviour.

Section 1.1.2 Laboratory culture

The animal can be cultured in the laboratory and grown on agar plate or in liquid culture supplemented with E.coli OP50 as a food source, which can be prepared in the laboratory. Making cultures of *C. elegans* is easy and relatively inexpensive.

Both the wild-type strain of *C. elegans* and mutant strains have experimental advantages. A good example of wild-type is N2, which was originally isolated from an English mushroom farm in the 1950's and is considered a standard model of *C. elegans* in the laboratory. Another good example of a naturally occurring wild-type is the Hawaiian strain, CB4856. This is a different wild-type strain, originally isolated from a pineapple in Hawaii. This strain is extremely important because it has a uniformly high density of single nucleotide polymorphisms (SNP) compared to N2 that assist the process of genetic mapping (Strange, 2006).

Section 1.1.3 Sex determination in *C. elegans*

C. elegans has two natural sexes (XO males and XX hermaphrodite), which have five pairs of autosomes and one pair of sex chromosomes each. Males are diploid for the five autosomes, but have only one X chromosome (XO). In contrast, hermaphrodites are diploid for all six chromosomes (Hodgkin *et al.*, 1979).

The reproductive system consists of a somatic gonad, the germ line and the egg-laying apparatus. Hermaphrodites can self-fertilise or mate with males, but cannot fertilise each other. When a hermaphrodite mates with males, the offspring is approximately 50% males and 50% hermaphrodite. In addition, there is a larger brood size in comparison to self-fertilisation. Mating with a male produces approximately 1000 offspring compared with hermaphrodite self-fertilisation which produces only around 300 offspring (Hodgkin and Branes, 1991). The reason for this is that the hermaphrodite produces more oocytes (during adulthood) than sperm (during L4 only) - thus the size of brood depends on the number of sperm available.

The adult hermaphrodite reproductive system consists of a symmetrically arranged bilobed gonad where each lobe bends, individual nuclei become partially enclosed by membranes of the oocytes, which progressively enlarge and mature and they pass down the oviduct (Wood, 1988). Mature oocytes can be fertilised by the hermaphrodite's own stored sperm or by a male's sperm, which they pass down through the spermatheca. Because of this, the male has a clearly visible fan shaped tail which is used to clasp the hermaphrodite during mating (Mee, 2002). During the first 30 minutes after fertilisation, the zygote develops a tough chitinous shell and a vitelline membrane. The eggs are then held in uterus until they are laid through the vulva around the gastrulating stage.

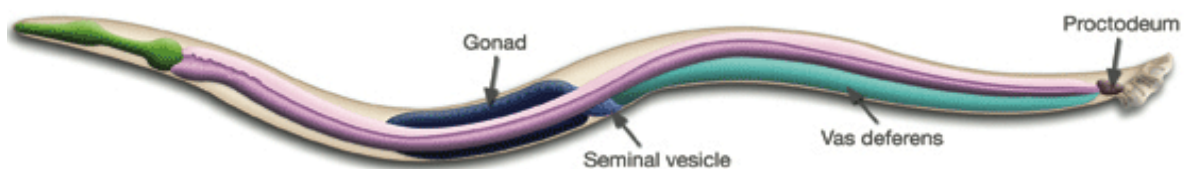
Section 1.1.4 Anatomy

Both sexes of *C. elegans* are unsegmented with a long cylindrical body which becomes narrow at the end (wormatlas.org). However the shape of the posterior is different between male and hermaphrodite. The male's posterior is fan shaped, whereas the hermaphrodite has a simple spike of a tail. *C. elegans* body consists of an outer tube (body wall) and inner tube (gut), which are separated by a fluid-filled body cavity or pseudocoel (Riddle, 1997; White, 1988). Between larval stages, the old cuticle is shed and a new stage specific cuticle is secreted (Kramer, 1997). The body wall consists of cuticle, hypodermis, excretory system, neurons and muscles. The underlying hypodermis consists of the pharynx, intestine and the gonad (in the adult). However, the approximate length of an adult hermaphrodite can range from 1mm to more than 3.5mm (Strange, 2006). The mature adult hermaphrodite worm has 959 somatic cells, whereas males have 1031 somatic cells.

The animal's neurons are located in the head and tail with the vast majority located in the head around the pharynx. The adult hermaphrodite has 302 neurons cells and 56 glial and support cells, whereas males have 381 neurons, 92 glial and support cells (Donald, 1997;

Horvitz, 1997; Wood, 1988). The muscle system is important for movement. *C. elegans* have four quadrants of muscle (two dorsal and two ventral), which are located down the whole length of the animal. Because of this, the animal can move forward and backward (Donald, 1997) as a result of muscle contraction and relaxation. Although *C. elegans* has a simple anatomy, it has large repertoire of behaviour. Good examples of this are locomotion, foraging, feeding, defecation, egg laying, and dauer larva formation. The sensory responses of touch, smell, taste and temperature as well as some complex behaviours like male mating can also be studied (Rankin, 2002; de Bono, 2002).

XO males



XX Hermaphrodite

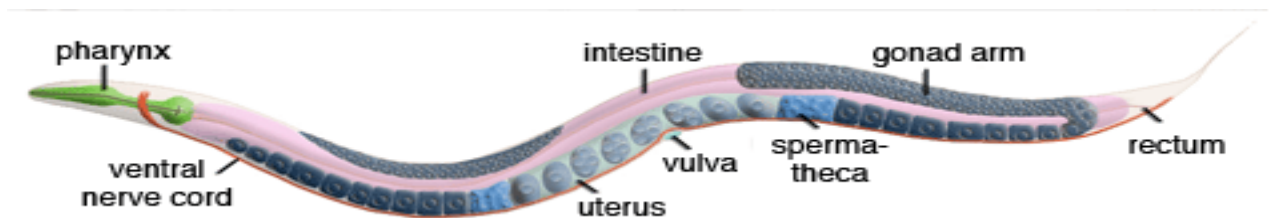


Figure 1.1: Photomicrograph showing major anatomical feature of XO males (above) and XX Hermaphrodite (below). (Figure is reprinted from www.wormatlas.org).

It can be seen from the figures that the *C. elegans* have an unsegmented, cylindrical shape and have a tip on the head and pharynx, which connects to the intestine. There are significant differences between the hermaphrodites and males in overall body size and structures. A good illustration of this is the somatic gonad and tail as can be seen from the diagram. The shape of their posterior is also completely different. In addition, the male is shorter and thinner than the hermaphrodite. However apart from these differences, the hermaphrodite's anatomy is almost the same as male's anatomy, such as the mouth, pharynx, gut and gonads.

Section 1.1.5 Life cycle

The life cycle of *C. elegans* is composed of 14-hours of embryogenesis followed by 36-hours of postembryonic development through four larval stages, called L1, L2, L3, L4 and adulthood at a temperature of 25°C (short time generation). Reducing temperature increases the time of development. The stage specific cuticle is synthesized at the end of the each larval stage and the old one is shed (Cassada and Russell, 1975). However, the time period between stages depends on temperature.

Section 1.1.5.1 Embryo

Embryogenesis has two stages, which are proliferation and organogenesis/morphogenesis (Sulston *et al*, 1983). In the first stage (proliferation), the cells divide from a single zygote cell to 558 cells. This stage is subdivided into two phases. The initial phase begins from 0 to 150 minutes where the zygote cell is formed from the production of embryonic cells, which takes place within the mother's uterus. The second phase starts from 150 to 350 minutes. In this phase, cell division and gastrulation is completed and the embryo is laid through the vulva into the surrounding medium (Bucher and Seydoux, 1994). The second stage (organogenesis/morphogenesis) takes from 5.5 – 6 hours to 12 – 14 hours. In this stage, different cells are produced and the whole body of the animal is subdivided into distinct tissues and organs. Embryogenesis takes place inside the egg, where the worm can roll around longitudinally (Sulston *et al*, 1983).

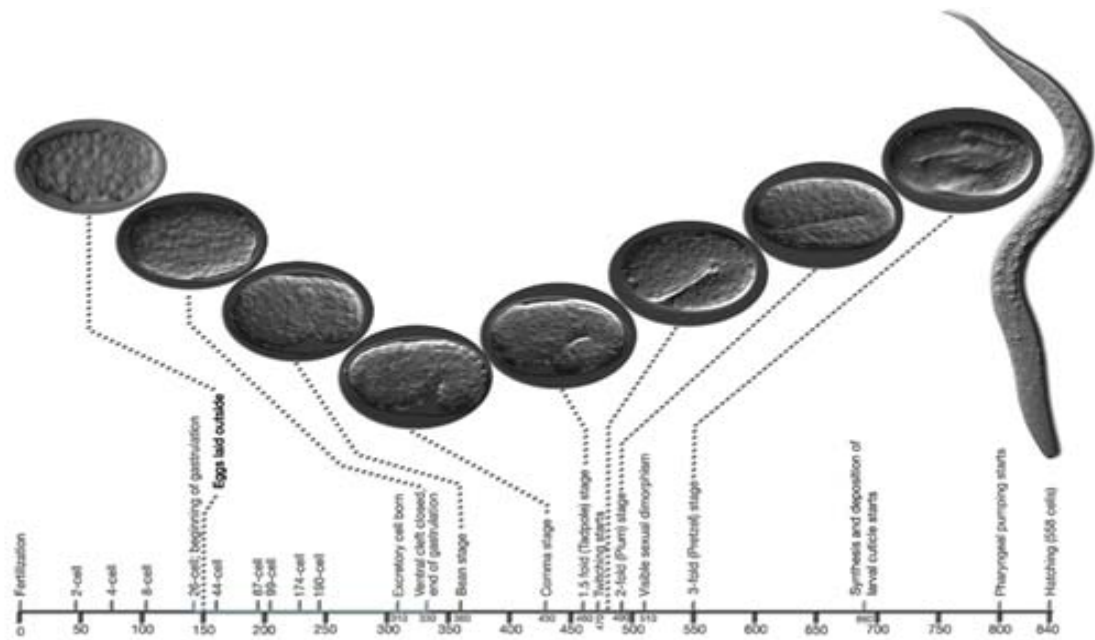


Figure 1.2: This figure shows embryonic stage of development at 22 °C (www.wormatlas.org).

The diagram shows embryonic development of the nematode *C. elegans* within the egg. The horizontal axis indicates the time in minutes after fertilisation at 22 °C during the period between 0 to 840 minutes. The embryonic stage starts directly after fertilisation. The first stage takes approximately 40 minutes. At 150 mins, the embryo has around 30 cells and is passed through the vulva. After that, the whole body of the animal is segmented into distinct tissues and organs. The animal hatches after around 840 minutes.

Section 1.1.5.2 Postembryonic development

Once the eggs hatch, they enter a stage called postembryonic development, which begins 3 hours after hatching (Ambros, 2000). However, before reaching adulthood, the *C. elegans* have to pass through four stages (L1-L4) separated by moults. The growth parameters of *C. elegans* lifecycle at 16, 20 and 25 °C are given in table 1.1. It was found that the worms die when incubated at temperatures above 25 °C. The L1 stage takes about 9 hours after fertilisation where the worm is approximately 250µm in length. In this larval stage, cell division begins when food is available. The second stage is L2, where the length of the worm increases, with some cell division and extension of tissue. Development of the gonad occurs in stage L3, and this stage is longer than L2. The final stage before the worm reaches adulthood is the L4 stage. In this stage the animal becomes sexually mature and sperm are produced by the hermaphrodite gonad only during this stage.

temperature (°C)	Embryogenesis (h)	Molts (h posthatch)				First eggs laid (h)
		L1-L2	L2-L3	L3-L4	L4-adult	
16	29	24	39	54.5	74.5	94-97
20	18	15	24	34	46	59-60
25	14	11.5	18.5	26	35.5	45-46

Table 1.1: This table shows the growth and lifespan of the *C. elegans* at 16, 20 and 25 °C in hours (Hirsh, *et al.*, 1976).

This table explains the time it takes to undergo each stage of development. According to table 1.1, *C. elegans* grow 2.1 times faster at 25°C than at 16°C, and 1.3 times faster at 20°C than at 16°C.

Section 1.1.5.3 Adult

This stage can be identified by the hermaphrodite worm's ability to produce eggs reflecting a switch in the gonad from sperm (L4) to egg (adult) production. The adult lays eggs for about 3-4 days. Over this period every adult can produce 300 self-fertilised progeny. They have a life-span of 10-15 days. The adult animal has many differentiated tissues, including muscle, a nervous system, gonad, hypodermis and gastrointestinal tract.

Section 1.1.5.4 Dauer

C. elegans also has an alternative L3 stage, which is called the dauer stage. This stage starts at the end of a modified L2 stage (Albert and Riddle, 1988). The dauer stage can be initiated by over-population or starvation. In addition, high temperature may also induce dauer larvae. The differences are that they are thin and their mouths are plugged therefore meaning they cannot eat. Despite this, dauer larvae can live for three months and can survive in poor conditions. Furthermore, this stage can move faster than the normal L3 stage when it is touched. Figure 1.3 shows the life cycle of the *C. elegans* at 22 °C (figure from Cassada and Russell, 1975).

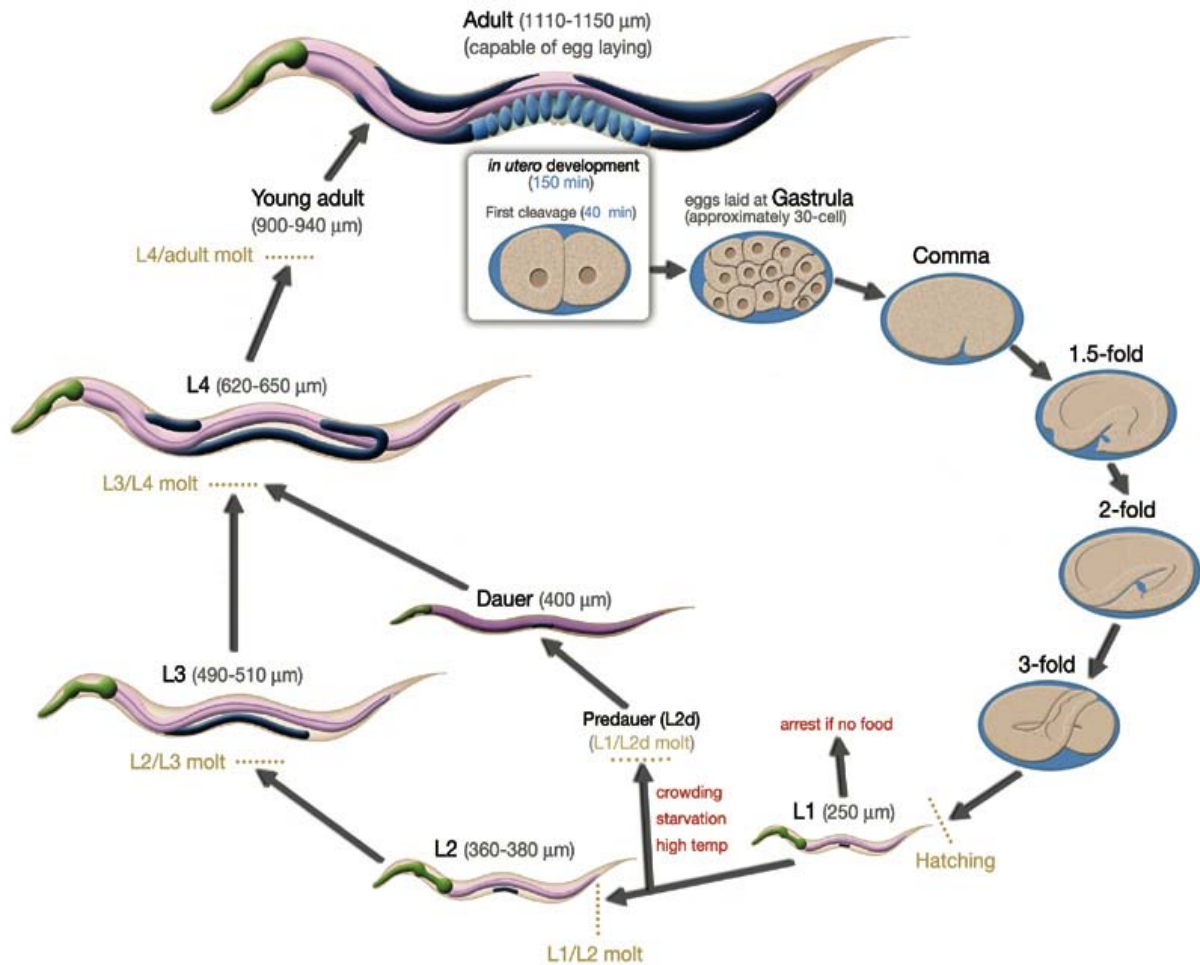


Figure 1.3: Life cycle of *C. elegans* at 22°C.

The figure shows the life cycle of *C. elegans* at 22°C from egg to laying eggs (adult). The blue numbers indicate the length of time between each larval moult. For example, eggs are laid after about 150 mins after *in utero* development. The length of the *C. elegans* at each stage of development is shown in micrometers (µm), next to the stage name.

Section 1.1.6 Benefits of selecting *C. elegans* for studying toxicity

The reason for using *C. elegans* as a model for toxicological testing is that they have an extensive list of qualities, which are important when selecting a test organism. Several good examples of this are reliability, relevance, responsiveness, reproducibility, abundance, geographical distribution and economics. In addition, the animal is a member of the phylum Nematoda, a ubiquitous, and diverse ecologically group of animals inhabiting both freshwater and marine environments as well as sediments and soils.

Previous research has shown that *C. elegans* can be provide useful measurements of toxicity when exposed to chemicals on agar plates (Williams and Dusenbery, 1988), in aquatic media (Williams and Dusenbery, 1990a) and in soil (Donkin and Dusenbery, 1994). *C. elegans* offer some advantages to ecotoxicological studies, in terms of their well-characterised genetics, development, behaviour and anatomy. The crucial aspects which make them good study models, as indicated by Hitchcock and Williams are that they have a similar size, age, health and reproductive ability which can be used for toxicological tests (Hitchcock and Williams, 1997). The genes of *C. elegans* have been shown to play a role in the mechanisms of toxicity, synaptic neurotransmission, identification of the key synaptic proteins and the regulation of synapses. Synaptic transmission in this animal is the most important form of communication between neurons. In *C. elegans*, synaptic vesicles merge with the presynaptic membrane in the synaptic junction at a specific active zone under a calcium regulated process in *C. elegans* (Nonet *et al.*, 1993). The majority of synaptic proteins in the *C. elegans* genome are highly conserved. In addition, mutants can be created, which gives the opportunity to study functional consequences *in vivo* (Richmond, 2005). Furthermore, *C. elegans* can be used to investigate the mechanisms of toxicity in multicellular organisms, for example in a forward genetic screen for mutations which gives resistance to certain toxins *e.g.* the resistance of *unc-17* mutants to lannate (Brenner, 1974). *C. elegans* are therefore a good model to study and understand the mechanisms of toxicity. *C. elegans* is helpful in RNAi studies by treating the worm with dsDNA or feeding with bacteria containing specific plasmids (Kamath and Ahringer, 2003) allowing the selective knock down of specific gene functions. By treating the worms with specific toxicants, toxic mechanisms can be linked with the under- or over-expression of genes (Lindblom *et al.*, 2001; Liao and Yu, 2005).

Section 1.2 Latrophilin

Section 1.2.1 Latrophilin/ CIRL (for Ca^{2+} -independent receptor of LTX)

The latrophilin protein is the target receptor for alpha-Latrotoxin (α -LTX) and is implicated in synaptic function. Although its signalling pathway is still unknown, this protein belongs to the secretin/ calcitonin family of G -protein-coupled receptors (GPCR). It is considered a new family of GPCR, known as a glycosylated protein and is a large (185 kDa) heptahelical receptor (Ushkaryov *et al.*, 2008). The receptor was isolated from bovine brain membranes by affinity α -LTX-chromatography and is called latrophilin (LPH) (Davletov *et al.*, 1996; Krasnoperov *et al.*, 1997). Latrophilin protein expression mostly occurs in neuronal or neuroendocrine tissues (Davletov *et al.*, 1996). As shown in figure 1.4, the protein contains three major domains, which include a long glycosylated extracellular domain, seven hydrophobic domains and a long cytoplasmic tail. Firstly, the extracellular domain contains an N-terminal signal peptide, a lectinlike sequence (~92 residues), galactose-binding lectin (GBL) region, the surface-attached extracellular matrix protein olfactomedin region (Snyder *et al.*, 1991; Loria *et al.*, 2004), and a hormone receptor motif (HRM) region. GBL is similar to galactose-binding lectin, and HRM might be involved in ligand binding (Rohou *et al.*, 2006). The HRM can also be found in other G-protein-coupled receptors (GPCRs).

Another part of the extracellular domain is a “Stalk” domain. This part is essential for proteolytic cleavage and G-protein coupled receptor proteolysis (GPS) (Change *et al.*, 2003). The extracellular domain is the actual GPCR proteolysis site (GPS) region (GPS), which is located in the cytoplasmic region that contains sequences which interact with intracellular proteins. This is an essential part for phosphorylation and palmitoylation. Moreover, it is important for receptor delivery to the plasma membrane (Kirill *et al.*, 2004). GPS is attached

to the membrane by an uncharacterised hydrophobic anchor (Volynski *et al.*, 2004) which may inhibit the release of the ectodomain into the medium. The second domain of Latrophilin is the seven transmembrane region (7TMR), which is similar to the corresponding regions of the secretin/calcitonin receptor family and It interacts with peptide hormones. α -LTX causes Ca^{2+} -dependent and -independent release of neurotransmitters. (Harmar, 2001).

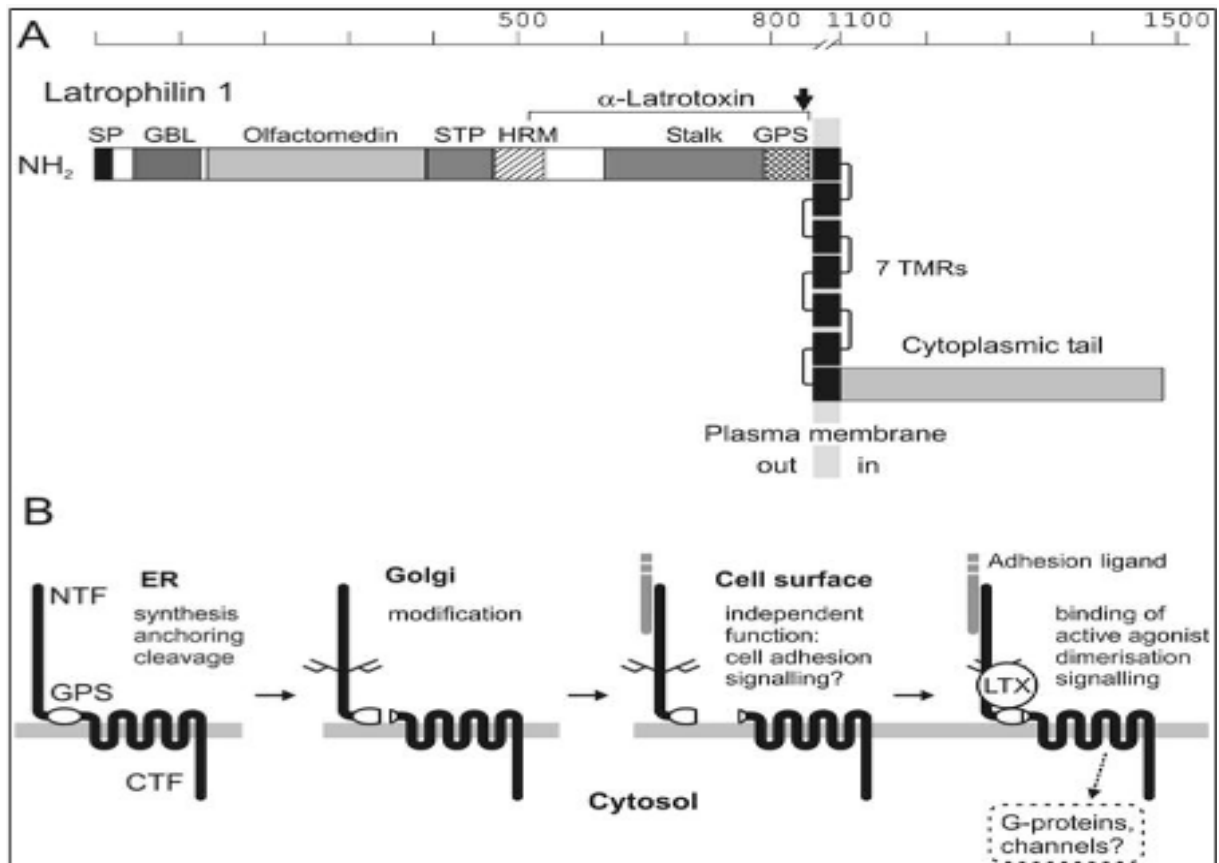


Figure 1.4: This figure shows the structural domains of latrophilin / C1RL1-3 and the activation of LHP receptor (Ushkaryov *et al.*, 2008).

Figure 1.4 A shows the structural domains of the receptor. It has large extracellular and intracellular domains. As described above, the extracellular domain contains five structure motifs. The homologous sequences of plasma membrane and intracellular domain have not been identified. The diagram shows where the GPS and HRM regions bind to α -LTX (Krasnoperov *et al.*, 1999). The lectin and olfactomedin regions could participate in adhesion interactions with the extracellular regions. The second domain is the seven transmembrane (7TMR), which is similar to the G-protein-linked receptors of the secretin family. Figure 1.4B shows that the 7TMR is attached to the cell membrane. The diagram also shows a long intracellular cytoplasmic tail at the C-terminal end of the LPH protein.

The structure of Latrophilin has only been recently characterised (Ushkaryov *et al.*, 2008). The members of this protein family, which includes 33 proteins, are classified as long N-terminus, group B (LNB) (Hayflick, 2000; Stacey *et al.*, 2000) or adhesion receptors (Fredriksson *et al.*, 2003). These are identified by their function/activity or through genome searches (Fredriksson and Schioth, 2005). All LNBs are similar to cell-adhesion proteins and signalling receptors, whereas the C-terminal fragments (CTFs) and the seven transmembrane regions are typical G-protein-coupled receptors (GPCRs) (Hayflick, 2000; Stacey *et al.*, 2000). N-terminal fragments and C-terminal fragments cleave at the GPS (proteolytic cleavage) (Ushkaryov *et al.*, 2008). The GPS cleavage is essential for binding between the two functional domains (N- and C-domain). The latrophilin receptor is formed of two protein components which are separated from each other and so behave as two different fragments. Despite this, the N-terminal fragment (NTF) strongly associates with the C-terminal fragments (CTF) upon solubilisation. Both components recombine on binding with α -LTX, facilitating signal transduction and activation of G-protein as well as stimulation of phospholipase C and subsequent calcium mobilization from intracellular stores (Volynski *et al.*, 2004) .

Although LPH has two homologues, latrophilin 2 and latrophilin 3 (LPH2 and LPH3), expressed in vertebrates (Ichtchenko *et al.*, 1998; Sugita *et al.*, 1998; Ichtchenko *et al.*, 1999; Matsushita *et al.*, 1999), only LPH 1 has been found to bind α -LTX with high affinity. Likewise, LPH 2 binds α -LTX, with a 10-fold weaker affinity compared with LPH1. LPH 3 is highly expressed in the mammalian brain (Sugita *et al.*, 1998). LPH1, LPH2 and LPH3 are also called CIRL1-3 (Ichtchenko *et al.*, 1999) or CL1-3 (Sugita *et al.*, 1998). All of them are G-protein coupled receptors, which have the same regional structure and contain various cell adhesion modules in their ectodomains. In addition, these receptors can transform

extracellular into intracellular signaling. Even in the absence of calcium in both LPH1 and LPH2 there can still be binding as described in the section 1.2.2. It was found that latrophilins are unusually large and the position of toxin binding to latrophilin 1 covered a large area, almost the size of the HRM, Stalk and GPS domains (Krasnoperov *et al.*, 2002).

Section 1.2.2 Mechanisms of α -latrotoxin action

Alpha-latrotoxin (α -LTx) is a large protein toxin (120 kDa), derived from black widow spider venom (BWSV), and is an extremely potent neurotoxic compound. α -LTx is a mostly hydrophilic protein that does not have classical hydrophobic signal peptide or transmembrane region (TMRs) (Ushkaryov *et al.*, 2008). The (α -LTx) causes massive secretion of transmitters at the synaptotagmin which binds tightly to neurexins (Petrenko *et al.*, 1991). It was found that the latrophilin / CIRL protein is the receptor of this toxin, (Mee *et al.*, 2004). This receptor is a calcium-independent receptor for α -LTx. The release of neurotransmitters can be both dependent or independent of Ca^{2+} when the latrophilin protein binds to α -LTx, but transmitter release is stronger in the presence of Ca^{2+} (Ushkaryov *et al.*, 2008). Although the influx of Ca^{2+} plays an important role in toxic response of LTX, latrophilin is the only protein that will bind to the immobilised α -LTx in the absence of divalent cations (extracellular). The main role of α -LTx in this biological system is that it binds to latrophilin protein 1 (LPH1), which primarily causes pore formation once the toxin has entered the plasma membrane (Finkelstein *et al.*, 1976). This causes the formation of Ca^{2+} channels. The release and influx of Ca^{2+} can lead to amplified pore- and receptor-mediated signals with the increase in $[\text{Ca}^{2+}]$ contributed to the mitochondria (Ushkaryov *et al.*, 2008). The next step is activation of receptor and intracellular signalling. However this signalling is a highly regulated process and cannot explain all the effects of α -LTx. Despite the fact that α -LTx induces its effects via the

latrophilin receptor, its signalling pathway, the toxin pore and some of the endogenous channels, are still unknown.

Transmitter exocytosis may only occur in the presence of calcium (Krasnoperov *et al.*, 1997). The presence of intracellular calcium is related to the intracellular activation of the receptor in the action of α -LTx and the type of neurotransmitter vesicle. It was shown that calcium increase in the membrane and the influx of Ca^{2+} is the trigger for fast action potential of intracellular events (Khvotchev, 2000). α -LTx promotes Ca^{2+} influx and stimulates exocytosis of dense-core vesicles containing noradrenaline, dopamine and neuropeptides. On the other hand, α -LTx stimulates exocytosis of the small clear vesicles that contain acetylcholine, glutamate and GABA in Ca^{2+} independent process (Khvotchev, 2000). High doses of α -LTx cause the stimulation of exocytosis and depletion of acetylcholine-containing vesicles in the absence of calcium. In contrast, neuropeptide-containing dense-core vesicles remain unchanged in the absence of calcium even during different actions at the neuromuscular junction. α -LTx can cause morphological deformation and cell death (Sudhof, 2001).

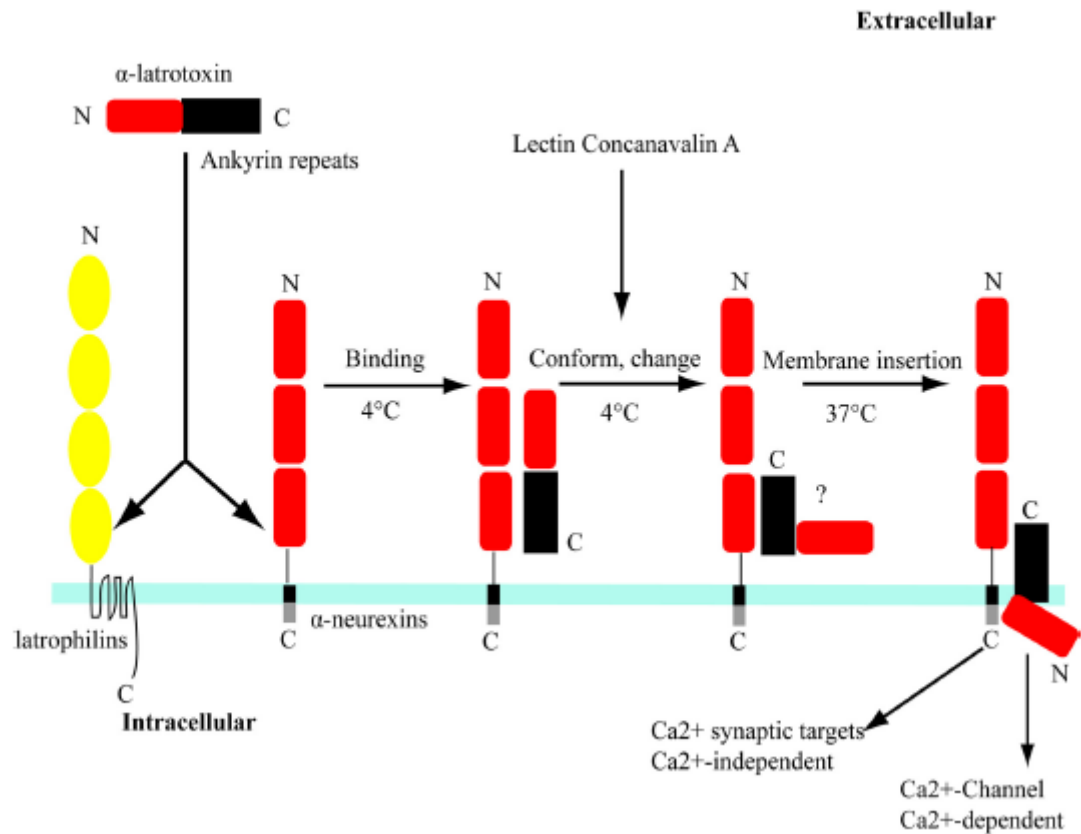


Figure 1.5: This figure shows the Mechanisms of alpha-latrotoxin action, which is taken from Sudhof(2001).

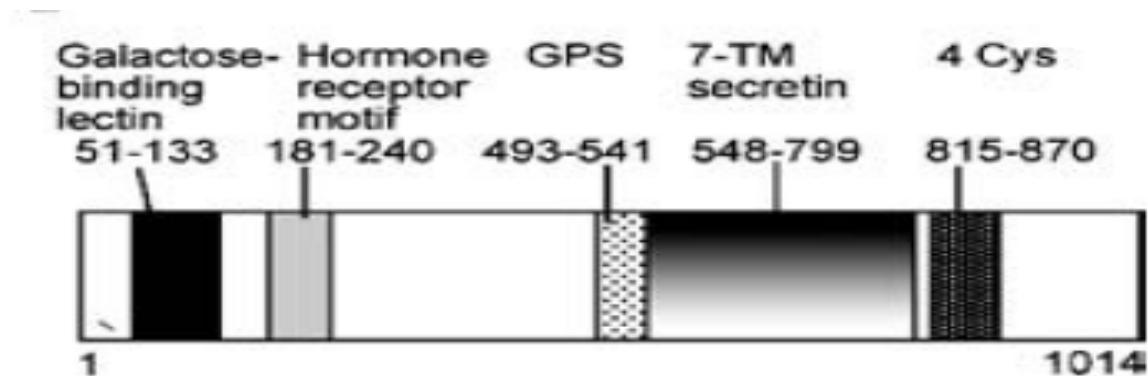
There are three models of stimulation of exocytosis by α -latrotoxin with receptors. These interactions are called membrane insertion, channel formation and intracellular activation of exocytosis. Initially, α -latrotoxin binds to a calcium-independent receptor (latrophilin/CIRL) and undergoes a conformational change with subsequent membrane insertion. Although receptor binding and the conformational change occur at 4°C, membrane insertion requires a higher temperature. The lectin concanavalin A (Con A) is responsible for this conformational change, although it does not inhibit receptor binding. The formation of Ca^{2+} channels and the translocation of the N-terminal domain of α -latrotoxin into the presynaptic intracellular space are caused by membrane insertion. The two processes of exocytosis which can be triggered are explained in section 1.1.2.

Section 1.2.3 Latrophilin: the *C. elegans* homologue

The latrophilin protein receptor in *C. elegans* is encoded by the B0457.1 (or *lat-1*) gene. There are two homologues of latrophilin in *C. elegans*, which were identified as B0457.1 (*lat-1*) and B0286.2 (*lat-2*) ([http://www.sanger.ac.uk/Projects/C. elegans](http://www.sanger.ac.uk/Projects/C._elegans)), whereas there are three latrophilin proteins in vertebrates. As shown in figure 1.6, the nematode protein has five conserved domains, which is 1014 amino acids in length (Mee *et al.*, 2004). The size of the protein is ~ 113KDa and is insoluble after expression in *E. coli* (Al-anizy, 2005). Although the *C. elegans lat-1(ok1465)* protein lacks an olfactomedin-like region, it has several common

N-terminal domains (hormone receptor motif, stalk, G-coupled protein proteolysis sites). The protein is more similar to vertebrate latrophilin than to its insect orthologues. The reason for this, as well as the LIT-binding ability of insect latrophilins, is currently unknown. As result of this, the latrophilin homologue from *C. elegans* has been studied to know about the function of these proteins and their role in regulating neurotransmitter release by latrotoxins (Mee et al., 2004; Willson *et al.*, 2004).

It was found that the latrophilin-dependent pathway for neurotransmitter release in *C. elegans* occurs when emodepside (an anthelmintic drug and an octadepsipeptide) causes paralysis. Therefore, the latrophilin receptor mediates the action of emodepside. This affects the pharynx, and coupling to G-protein ($Gq\alpha$) and phospholipase C ($PLC-\beta$) via the latrophilin-signalling pathway which regulates the mechanism of effect of emodepside (Willson *et al.*, 2004). Moreover, the *UNC-13* protein is required by the latrophilin signalling pathway to stimulate neurotransmitter release by vesicular exocytosis.



Figures 1.6: This figure indicates the structural domains of latrophilin (*lat-1*) (Mee *et al.*, 2004).

The nematode protein has five domains, which contain 1014 amino acids. These domains were identified by manual alignment with the rat latrophilin sequence. The black box on the left of the diagram shows a galactose-binding lectin domain (51-133), which is followed by the hormone receptor motif (181-240). Between 493-541, is the GPS motif and between 548-799 is the seven-transmembrane domain (7-TM secretin) is shown by a box with black to white fade. Finally, a black box with white dots shows the four conserved cysteine residues (815-870).

Section 1.2.4 *Lat-1(ok1465)* (ok1465) worms

An extensive deletion of the LPH gene is called ok1465. Worms containing this allele are designated strain VC965, which are heterozygous for the ok1465 deletion (<http://aceserver.biotech.ubc.ca/cgi-bin/generic/allele?class=Allele;name=ok1465>). Then they were used to generate homozygous worms with a deletion in the LPH protein (ok1465 null worms) (DRB, unpublished data). The deletion of *lat-1(ok1465)* in the ok1465 allele is between bases 18185 and 20395 of the B0457 cosmid sequence. This deletion leads to the removal of exons 3a to 5 of the *lat-1a* transcript (B0457.1a) resulting in the splicing of exon 2 and exon 6 as shown in figure 1.7 (Guest, 2007). As mentioned in the previous section, the structure of the wild-type protein has five conserved domains, but the deletion from the *lat-1(ok1465)* are between amino acids 100 to 500, a significant region of the extracellular domain and the first transmembrane helix of the 7-transmembrane domain. As a result, the latrophilin protein is likely to be dysfunctional. The evidence of dysfunction is that the homozygous null allele of the *lat-1(ok1465)* gene (allele ok1465) causes 97% of lat/lat worms to die during embryogenesis or early larval stages, demonstrating an essential role of *lat-1(ok1465)* during early development of the worm. It was found that 33% of *lat-1(ok1465)* (ok1465) homozygous offspring died at embryonic stage and 65% died at larval stage (Adenle, 2008). As a result, only 2-3 (2%) homozygous offspring from *lat-1(ok1465)* (ok1465) hermaphrodites complete their development to an adults (Adenle, 2008). In contrast, 0% death was found in wild type worms (N2), with all the offspring surviving to adulthood. The reason for lethality of the *lat-1(ok1465)* (ok1465) worms is the failure of gut attachment to the pharynx in the larvae stage and failure of epithelial migration. A second dysfunction affects the defecation cycle. The defecation cycle of N2 is 45 ± 3 seconds per defecation cycle, whereas for *lat-1(ok1465)* worms is 80 ± 10 seconds per defecation cycle. *lat-1(ok1465)*. It is unknown whether these effects (low offspring number and prolonged defecation) are both due

to *lat-1(ok1465)* gene deletion or other effects during the process of mutagenesis. However, studies showed that microinjection of the full length *lat-1(ok1465)* gene into the mutated *lat-1(ok1465)* animal (ok1465) rescued these phenotype problems. The number of offspring then improved and the defecation cycle time became shorter (Adenle, 2008).

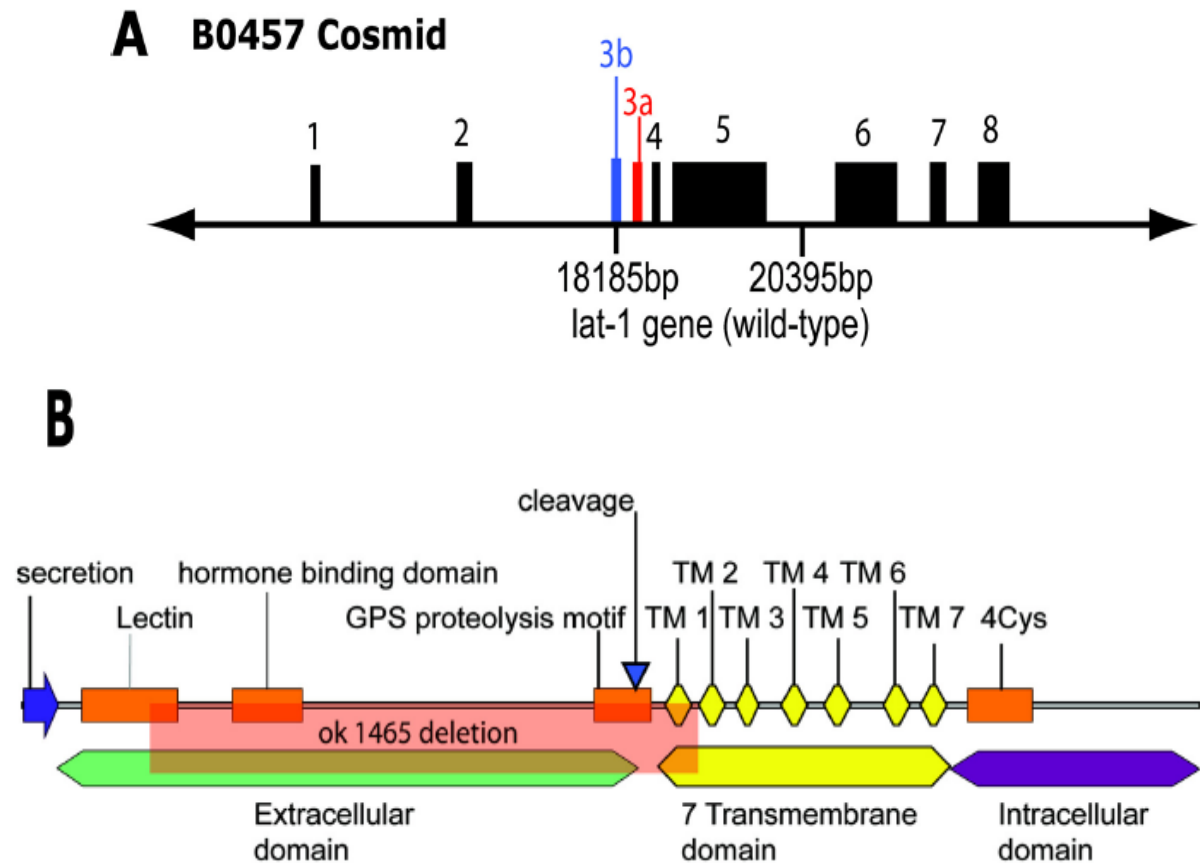


Figure 1.7 shows diagram of the ok1465 gene (taken from Adenle, 2008).

It can be seen from the diagram that the wild-type *lat-1(ok1465)* gene, in the B0457 cosmid, has exons 1-8 of the *lat-1(ok1465)* gene worms, which are illustrated in black boxes, with exons 3a and 3b, drawn in red and blue respectively. The introns are shown as black lines at 18185 and 20395 bp. *Lat-1(ok1465)* worms have a deletion that spans these two introns. The deletion, from exon 3b to exon 5, is shown in figure 1.7A. From figure 1.7B, the deletions of amino acids in ok1465 worms can be seen. Moreover, the diagram shows that the deletion of the significant region of the extracellular domain and the first transmembrane helix of the 7-transmembrane domain has occurred. The green colour shows the extracellular domains, the yellow colour shows the seven transmembrane and the purple colour indicates the intracellular domains (C-terminal).

Section 1.3 Mutagenesis

Section 1.3.1 Choice of Mutagen

Point mutations and chromosomal rearrangements are different outcomes of mutagenesis. Point mutations are the most common type of mutation, which can be delimited by localized changes such as, transitions, transversions, or nucleotide additions or deletions (one or a few nucleotides). A point mutation is also called a base substitution and occurs when a single nucleotide is replaced with a different nucleotide. This kind of mutation is caused by random mutations (EMS, TMP or UV) although most point mutations are caused by EMS (Donald, 1997). The other mutagenic method is to induce chromosomal rearrangements, which involves duplications (tandem, insertional, or free), deficiencies (deletions of DNA sequence), inversions, translocations, and more complex combinations of events. Although rearrangements can be generated by some chemical mutagens, they are often induced by irradiation. Point mutations should be used when effective induction of loss-of-function mutations in single genes is required. If the point mutations are actually small deficiencies, polymerase chain reaction (PCR) can be used to determine this mutation. On the other hand, chromosome rearrangement should be used, when larger mutations such as multigene deficiencies or chromosomal balancers are needed.

In order to generate point mutations, EMS is often used, which is an efficient mutagen. Although EMS mainly causes G/C-A/T transitions, it can often lead to some small deletions and other chromosomal rearrangements (Anderson, 1995). Another cause of transitions and transversions, as well as some small deletions and other chromosomal rearrangements, is ENU. To prevent isolating strains, which have more than one mutation, two criteria should be applied. Firstly, only the minimum amount of mutagen required to achieve the essential

results should be used. Secondly, a mutagenized animal should be backcrossed to obtain an unmutagenised background and the desired mutations resegreated (Anderson, 1995).

Section 1.3.2 Application of Mutagens

The best stage for mutagenesis is between L3/L4. The reason for this is that the number of germ-line nuclei is at its maximum, but they are still mostly mitotic. This allows several replications to intervene between the mutagenic treatment and gamete formation which may be extremely important for the fixation of mutation. A mutation in one of the germ-line nuclei may be propagated to many gametes, because the L4 larva has a small number of rapidly dividing germ-line nuclei. L3 and L4, however, have all their sperm and also have oocytes in various stages of maturation. Mutagenising younger worms (L3/ L4) increases the probability of “jackpots” or many mutants increasing in one event. When adult hermaphroditic worms are mutagenised with EMS (Johnsen and Baillie, 1991), there are no jackpots.

When the worms are mutated by mutagenesis, every generation needs to be considered from P0 to F2. When P0 is treated with EMS, every sperm and every ova will have different mutations. This means every F1 is unique, because all the mutations come from the sperm and ova. Each mutation will be clonally expanded by self-fertilisation as seen in figure 1.8. Therefore, the key effect is the number of F2, which will be screened. Only a selection of the worms from the F1 require screening, because 25% of the worms within that group of offspring will carry the same mutation in homozygous form. As result of this, every F1 worm carry one mutation in one gene. The same applies in the F2 generation. For example, F1 may have gene X^*/X , where gene X is the wild-type copy. In F2, the offspring will be 25% X/X (homozygous), 50% X^*/X (heterozygous) and 25% X^*/X^* (homozygous) as seen in figure 1.6. Only the X^*/X^* (mut/mut) animals will show a change in phenotype, because most of the mutations are recessive. However, N2 gives rise to huge number of animals. After bleaching

the wild type worms from the same hermaphrodite, one out of every four animals in the F2 generation, should be homozygous. Thus, there is a chance to see a new phenotype. On the other hand, this cannot be possible for the *lat-1(ok1465)* worm, because every individual produces 4 ± 4 surviving adults. This is a great difficulty because there is only a 25% chance of getting a recessive gene in homozygous form. This might mean that these three offspring might have the X/X gene not the X*/X* gene. When bleaching *lat-1(ok1465)* worms, four worms from the F1 generation were required to get four worms in the F2 generation, of which one worm should have the X*/X* (m/m) gene. Millions of F1 eggs for *lat-1(ok1465)* worms are required because most of eggs do not survive to adulthood. Therefore, a huge number of *lat-1(ok1465)* worms should be screened to find mutations which lead to an increased brood size.

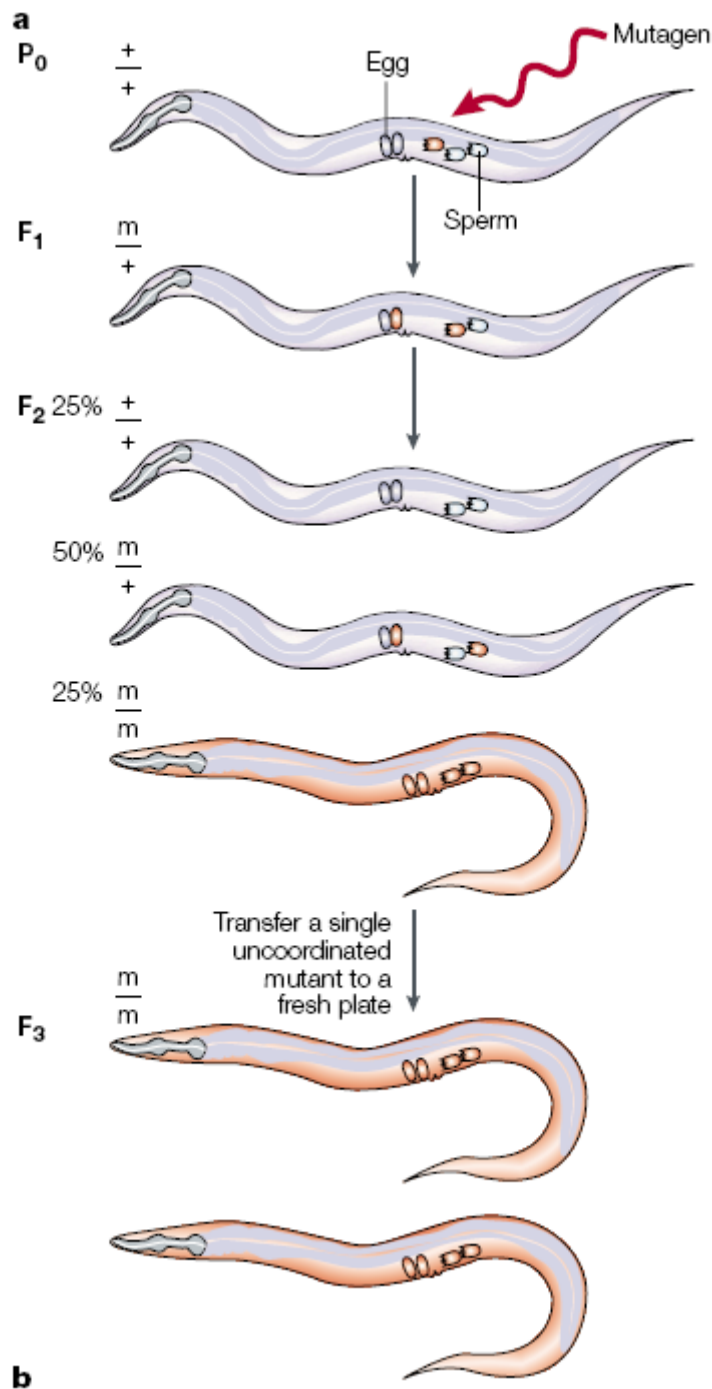


Figure 1.8 how the mutagen affects every single worm:

The parental generation (P) was treated by 50 mM of ethyl methanesulfonate (EMS) (Jorgensen.E.M and Mango.S.E) (taken from Nature Reviews Genetics). F1 is the first generation and F2 is the second generation.

Figure 1.8 shows that how the mutagen such as EMS affect the worm. When the worms (P0) will be treated with EMS, it will induce mutations in every sperm and each over (mutated germ cells are indicated in red). F1 heterozygous hermaphrodites are generated by mutating P0 with EMS and self to get F2 generation. Then F2 recombinants were isolated on to individual plates. It can be seen from figure 3.6 that there is just 25% of X^*/X^* (homozygous) in F2 generation. This means that the wild type has 1 in 4 F2 will be homozygous. From the figure 1.8 it can be estimated that four F1 worms should be mutagen and four F2 should be screen to get as N2 chance (25% of X^*/X^* (homozygous)).

Section 1.3.3 Mechanism of EMS mutagenesis

EMS (ethane methyl sulfonate; or methanesulfonic acid ethyl ester) is a mutagen that induces direct mutations in DNA by nucleotide substitution. Although EMS is extremely dangerous and is a suspected carcinogen, it is one of the most powerful means available, when compared to mutants produced using UV or TMP. Therefore, EMS is a common strong chemical mutagen for *C. elegans* (Brenner, 1974). 50 mM EMS mutagenesis can affect an average sized gene, which is between 1×10^{-4} bp and 5×10^{-4} bp (Brenner, 1974; Meneely and Herman, 1979). The average molecular mutation frequency per animal varies per locus from one mutation every 61 to 192 kb with an average of one mutation every 104 kb (Cuppen, 2007).

EMS can cause point mutation in DNA by the transition of a G base to a T as can be seen in figure 1.9. A transition, is where a purine is exchanged for a purine (A ↔ G) or a pyrimidine for a pyrimidine, (C ↔ T). A transversion occurs when a purine is exchanged for a pyrimidine or a pyrimidine for a purine (C ↔ A/G) (Loechler *et al.*, 1984; Snow *et al.*, 1984). Moreover, it generates G/C to C/G transversion and A/T to G/C transitions. About 6.8% of the mutations are not G/C to A/T, which is well within range with forward (phenotype-driven) genetic data in *C. elegans* (Cuppen, *et al.*, 2007). It was found that 13 out of 238 EMS-induced single base pair mutations (5.5%) are not G/C to A/T (Anderson 1995). Point mutations that occur within the protein coding region of a gene may be classified into three kinds, known as silent mutations, missense mutations and nonsense mutations.

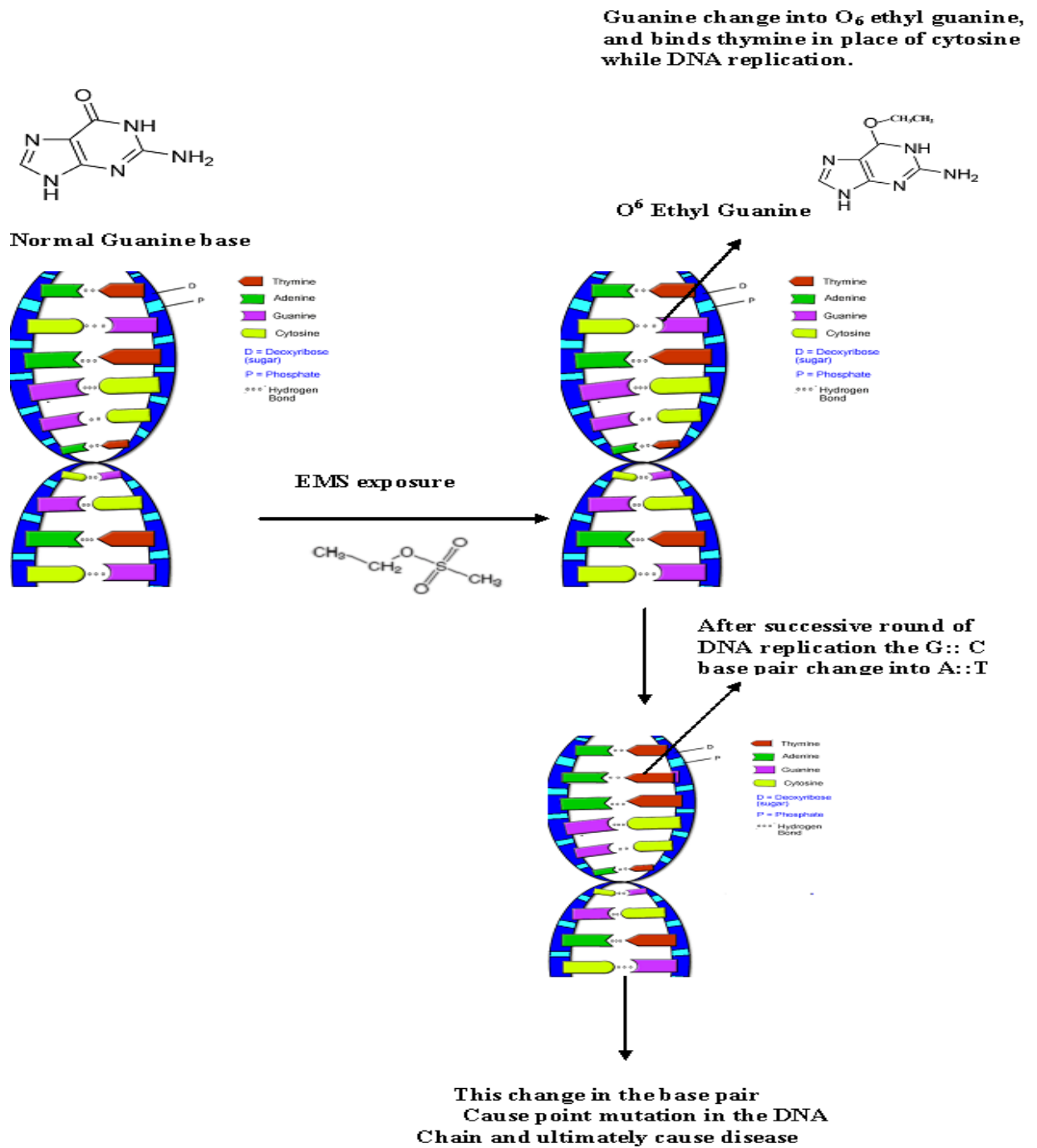


Figure 1.9 :This figure shows the Mechanism of mutagenesis by EMS.

EMS ($C_3H_8O_3S$) cause random mutations by nucleotide substitution (cause guanine alkylation) and produce point mutation within the DNA chain. The ethyl group of EMS reacts with guanine in DNA, forming the abnormal base O-6-ethylguanine. During the replication process, this abnormal O-6-ethylguanine base binds with thymine in place of cytosine and after many rounds of replication the G::C base pair changes into A:T base pair and can cause point mutation in the DNA chain. This base pair change is always harmful to the cells.

Section 1.4 Aims of study

Latrophilin (*lat-1*) protein is a member of class B family of G-protein coupled receptors as described in section 1.2. The signalling pathways of the latrophilin protein are poorly understood. *C. elegans* have been used to understand a wide range of latrophilin protein functions and mechanisms. Induced mutations are necessary to study the genomic functions of *C. elegans*. Therefore, mutagenesis of *lat-1(ok1465)* worms was required to further study the function of the *lat-1(ok1465)* gene. Using random chemical mutagenesis by EMS this study seeks to detect single nucleotide mutations in genes that can increase the brood size of *lat-1(ok1465)* worms.

2. Materials and Methods

2.1 Materials

All solutions were made up to 1 litre with UHP water and sterilized by autoclaving.

Storage was at room temperature unless otherwise stated.

Table 2.1.1 Nematode Growth Medium (NGM) agar

NaCl	3.0 g.l ⁻¹
Agar	17.0 g.l ⁻¹
Peptone*	2.5 g.l ⁻¹
Cholesterol	5.0 mg.l ⁻¹

* In enriched or 'high peptone' NGM plates, the peptone concentration was 20 g.l⁻¹

After being autoclaved NGM was allowed to cool upto 60°C. Under sterile conditions the following were added, to give a final concentration of:

CaCl ₂	1 mM
MgSO ₄	1 mM
Potassium Phosphate Buffer	40 mM

The potassium phosphate buffer (1M, pH 6.0) was prepared by titrating basic K₂HPO₄ (1M) with 500 ml acidic KH₂PO₄ (1M) until pH 6.0 was reached. The resultant solution was then made upto 1 litre with ultra pure water, dispensed into 150 ml aliquots, autoclaved and stored at 4°C.

Table 2.1.2 Potassium Phosphate Buffer (pH 6.0)

1M KH ₂ PO ₄	434ml
1M K ₂ HPO ₄	66ml

The above volumes mix to make the buffer pH6.

Table 2.1.3 LB Broth – made up using 25g of a ready mix.

Tryptone	10g
NaCl	10g
Potassium Phosphate Buffer	5g

Table 2.1.4 LB Agar – made up using 35g of a ready mix.

Tryptone	10g
NaCl	10g
Yeast extract	5g
Micro Agar	10g

Table 2.1.5 K-Medium

KCl	32 mM
NaCl	53 mM

Table 2.1.6 Egg Isolation Solution

NaOCl (stock at 13%)	3ml
NaOH (1M)	6ml
H ₂ O	15ml

* Stock is at a concentration of 13.5 to 16% (Aldrich, Dorset, United Kingdom) Due to the tendency of the sodium hypochlorite to break down over time, the Egg isolation solution was made up fresh monthly and stored at 4°C.

Table 2.1.7 Freezing down Solution

Glycerol	300 g.l ⁻¹
NaCl	5.84 g.l ⁻¹
Potassium Phosphate Buffer (pH 6.0)	50mM

The potassium phosphate buffer (1M, pH 6.0) was prepared by titrating basic K₂HPO₄ (1M) with 500 ml acidic KH₂PO₄ (1M) until pH 6.0 was reached. The resultant solution was then made upto 1 litre in a volumetric flask with ultra pure water, dispensed into 150 ml aliquots, autoclaved and stored at 4°C. The solution was autoclaved, and then sterile MgSO₄ to a final concentration of 0.3 mM was added.

Table 2.1.8 Lysis Buffer – made up to 100ml as described.

Tris-HCL (pH 8.0) –	1ml
KCl	0.37275g
MgCl ₂	0.0508275g
IGEPAL (Nonidet P40)	450µl
Tween 20	450µl
Gelatin	10µg
Protienase-K (Stock 10mg/ml)	500µl (to give 50µg/ml)

Proteinase-K must be kept frozen so once added, 10µl of the solution were aliquot into 500 µl PCR tubes and stored at -20°C.

Table 2.1.9 12% TBE-PAGE Gel – made up to 100ml as described.

Polyacrylamide (30%)	40ml
H ₂ O	50ml
10 x TBE	10ml
TEMED	100ul

This solution was kept at 4°C, and the following was added when assembling a gel.

Ammonium persulphate (Stock 0.1g/ml) - 10µl per ml of 12% TBE page gel.

Ammonium persulphate (APS) stock was stored in 1.5ml centrifuge tubes at -20°C.

Table 2.1.10 TBE – this was made up as a 10X concentrate. 100ml of the concentrate was then made up to 1 liter.

Tris	108g
Boric Acid	55g
0.5M EDTA (pH 8.0)	40ml

Table 2.1.11 PCR Master Mix – Ready to use, each vial contains 1.8ml at a 1.1x working concentration. Thermoprime plus DNA polymerase – 1.25 units.

Tris-HCL (pH8.0)	75mM
(NH ₄) ₂ SO ₄	20mM
MgCl ₂	1.5mM
Tween 20	0.01% (v/v)
dATP, dCTP, dGTP and dTTP	each at – 20mM

The master mix was stored at 4°C.

Table 2.1.12 Loading Buffer – made up to 10ml

Glycerol	5ml
Bromophenol blue	25µl
Xylene cyanol FF	25µl
10 X TBE	1ml

2.1.13 Primers – made as per company instructions to 100pm.

Lat1 ok1465 null 5'

5' - AGC TTG GTC AGG GTA GTA CT – 3'

Lat1 ok1465 null 3'

5' GGT TTT TGG TCC AAA TTT CGA CC – 3'

Lat1 ok1465 wild-type 5'

5' – TTC ACA TTC ATT GTG ATG GA – 3'

Lat1 ok1465 wild-type 3'

5' – TCC GGT AAT CCA GAG ACA TC – 3'

To avoid repeat freeze-thawing, primers were aliquoted (15µl) into PCR tubes and stored at -20°C.

Table 2.1.13 M9 Buffer

KH ₂ PO ₄	3 g
Na ₂ HPO ₄	6 g
NaCl	5 g
MgSO ₄ (1M)	1 ml
dH ₂ O	1 L

Table 2.1.14 S. Basal

NaCl 0.1 M	5.8g
Potassium phosphate (pH6) 0.05 M	50ml
Cholesterol (5 mg/ml in EtOH) 1 ml/800ml	1ml

The mixture must be autoclaved.

Table 2.1.15 S Medium

S. basal	1 litre
1M potassium citrate pH6	10ml
trace metals solution	10ml
1M CaCl ₂	3ml
1M MgSO ₄	3ml

Table 2.1.16 EMS

50mM EMS	20μ+4ml of M9 buffer
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Supernatants of worms treated with EMS were inactivated by mixing them with an equal volume of sodium thiosulfate (0.1M NaOH, 20% w/v Na₂S₂O₃) for 24 hours.

2. Methods

Section 2.2.1 *E.coli* Cultures

LB agar plates are prepared using Tryptone, NaCl and Yeast extract as mentioned in the table 2.1.4. A single colony of OP50 *E.coli* has been streaked onto a LB agar plate and incubated at 37°C overnight. Preparation of Luria Bertani medium (LB broth) was done as shown in table 2.1.3. 5ml of LB broth was incubated with a single colony of *E.coli* from a streaked plate (in a class II hood) and incubated overnight at 37°C with rapid shaking at 240rpm. Next day, this mixture was added to 500ml of LB broth and incubated overnight at 37°C and 240rpm. The *E. coli* OP50 solution was then ready for use in seeding NGM plates. The *E. coli* OP50 streaked plate and *E. coli* OP50 liquid cultures were stored at 4°C for several months.

Section 2.2.2 Gel electrophoresis

- 1) The gel solution (TBE-PAGE gel) was prepared as described in material section.
- 2) The glass plates and spacers were cleaned using hot water and soap then they were set them aside to dry.
- 3) After addition 5µl of TEMED to the 6ml gel solution, 5ml of this gel was poured into a gel casting mould. The appropriate comb was then inserted into the gel, before the acrylamide polymerizes.
- 4) The acrylamide was allowed to polymerize for 30 minutes at room temperature before the comb was carefully pulled out. The excess acrylamide was removed with tissue paper and the gel was placedp in an electrophoresis tank.
- 5) 1x TBE (running buffer) was added to the tank so that it covered to cover the wells properly.
- 6) Loading buffer was added to each of the DNA samples and gently mixed.

- 7) These samples and 100bp DNA ladder were carefully loaded into the wells of the gel.
- 8) The gel was run for 1.5 hours at 100V.
- 9) The gel was then removed from between the glass plates and placed in a solution of 0.0001% ethidium bromide in purified H₂O for 30 minutes and washed twice with H₂O for a further 10 minutes.
- 10) A UV trans-illuminator was used to visualize the fluorescence on the gel. The bands were then photographed by using Bio Rad Gel-Doc system using Quantity One software.

Section 2.2.3 C.elegans Methods

Section 2.2.3.1 Growth and Maintenance of *C.elegans*

When *C.elegans* was first investigated by Brenner (1974), *Caenorhabditis elegans* were maintained on Nematode Growth Medium (NGM) agar. They can be kept at temperatures between 15°C and 25°C, which affects the rate of growth at development. NGM agar was prepared using the following reagents; 3g NaCl, 17g agar, 800µl of 5mg/ml cholesterol in ethanol and 2.5g peptone were dissolved in 800ml of distilled water. This was then autoclaved for 50 mins. The mixture was microwaved for 10-15 minutes until molten before allowing the flask to cool in a 58°C water bath for 45mins. The following reagents were also heated to 58°C (700µl 1M CaCl₂, 700µl 1M MgSO₄, 20ml 1MKP0₄ buffer PH) before being added to the mix. NGM solution was spilled into 3cm, 4.5cm or 9cm petri plates under sterile conditions in a second class hood. Then, *E. coli* OP50 was seeded in plates. A flamed glass spreader was used to evenly spread the *E.coli*. After that, the plates were left to air-dry under sterile conditions. The plates then were incubated overnight at 37°C and stored at room temperature. The plates can be stored for 2-3 weeks at room temperature.

Section 2.2.3.2 Worm Harvesting

- 1) When a plate had more adults, it was ready to harvest.
- 2) Worms were washed off the culture plates with 5-10mls of M9 buffer or K-medium and transferred into 50ml centrifuge tubes.
- 3) Worms were spun down at 1500 rpm (low speed) for 1 minute then the supernatants were removed.
- 4) Step three was repeated to remove as many bacteria as possible.
- 5) The K-medium was removed and the last 2 ml (the worm pellet) was left. The worms were then ready for bleaching.

Section 2.2.3.3 Preparation of synchronous cultures

This method is important for the removal of contamination or egg isolation.

- 1) Worms were harvested as described in section 2.2.3.2.
- 2) Fresh bleaching solution (2 ml sodium hypochlorite, 500 μ l of 10M NaOH, and 2.5 ml of deionised H₂O, mixed by vortexing) was made up.
- 3) The bleaching solution (4 ml) was added to 2 ml of the worm pellet in a 15 ml centrifuge tube.
- 4) The tube was vortexed for 4 mins.
- 5) The progress of the bleaching was checked by placing a drop of worm mixture on to a glass slide and observing it under a dissecting microscope. When the worms begin to show signs of breakage (embryos spilling out), the next step should be started immediately. If not, bleaching for a further minute should be conducted.
- 6) The tube was then spun in a centrifuge for 1 minute at 1500 rpm.
- 7) The supernatant was carefully discarded ensuring not to disturb the pellet and a fresh volume of ice cold K medium was added to fill the centrifuge tube.

- 8) Steps 5, 6 and 7 were repeated for three further spins to clean the eggs of bacteria and bleaching solution.
- 9) After the final spin has finished, the supernatant was removed and the final 1ml of K medium and pellet (eggs) was transferred to a 6-well plate using a sterile Pasteur pipette.
- 10) The 6-well plate is sealed with laboratory film and incubated at 15°C overnight for the eggs to hatch into larvae. The reason for leaving the larvae without bacteria was to be sure that the worms remain at the L1 stage.
- 11) The worms were transferred to a plate for synchronous growth.

Section 2.2.3.4 Freezing worm stocks

C.elegans can be frozen and stored at -80°C. Starved worms from the L1 stage are the most likely to survive the freeze/thaw process. On the other hand, a well fed adult or egg will not survive the freezing. Therefore, plates must be allowed to run out of food, so that plenty of L1s and L2s are present. For example, if the worms are grown at 15°C, they will be ready to freeze in 1 week. Otherwise, adults were bleached as described in section 2.3.4 and hatched on plates without bacteria to get L1 larvae.

- 1) This plate was washed with 5 mL of S basal medium.
- 2) The liquid was carefully discarded ensuring not to disturb the pellet.
- 3) An equal volume of freezing solution 500µl was added to the 500ul worm pellet in S basal medium and mixed by inverting several times.
- 4) 1ml of worm mixture was aliquoted into labelled cryotubes and stored at -80°C for future use.

- 5) The worms were checked the next day to ensure a successful freeze. This was done by transferring an aliquot of frozen worms to a NGM plate to ensure enough worms have survived.

Section 2.2.3.5 Transferring worms grown on NGM plates

There are three methods used for transferring *C.elegans* from one petri plate to another. Firstly, the easiest and fastest method is the chunking method. A scalpel is sterilized using a flame then allowed to cool. It is then used to transfer a chunk of agar from the old plate to the fresh plate where they feed on *E.coli* OP50. The worms will move from this chunk and spread out onto the bacterial lawn of the new plate. Although this method is fine for transferring homozygous stocks, it is not recommend for heterozygous population which must be maintained by mating. Another method is that the worms are washed from an old starved plate with sterile water or K.medium. The liquid is then transferred into 15 ml centrifuge tube and placing on ice for 5-10 mins. When the worms have settled at the bottom of the tube, the water is carefully removed without disturbing the worm pellet. The worm pellets are then distributed onto fresh plates and left until dry in class II hood. Finally, the third method uses a worm picker to pick a single animal from one plate and transfer it to another. The worm picker can be made by mounting a 1-inch piece of 32 gauge platinum wire into the tip of a Pasture pipette. Platinum wire was heated and left until cool. The worm picker should be flamed between transfers to avoid contaminating the worm stocks.

Section 2.2.4 Assays for developmental and behavioral phenotypes in *C. elegans*

Section 2.2.4.1 Measurement of brood size for N2

Day 1

- 1) A plate with enough gravid adults worms was bleached as described in section 2.2.3.3.

Next day

- 2) The L1 worms were incubated at 20 °C, until they reach the L4 stage, which occurs on day three.

Day 4

- 3) 10 L4 hermaphrodites were transferred individually to a 3 cm plate (one worm/plate), until they had matured and laid the first few eggs.

Day 5

- 4) The first day where eggs were seen on the plate, was defined as the first day of laying eggs.

Day6

- 5) The 10 worms were transferred individually to a new plate to prevent overcrowding and to make sure the number of progeny is related to one mother over three three days

Day7

- 6) The 10 mothers were transferred to a new plate as well.

Day8

- 7) The 10 mothers were transferred individually to a new plate again. If there are still eggs on the plate, the adults should be moved again to another plate, until egg-laying has stopped.

- 8) 3 days after removal of the parents from each plate, the number of progeny was counted using the worm pick, and all single adults picked up were flamed to avoid double counting.

Section 2.2.4.2 Microscopy of *C. elegans*

Brood size, worm picker and developmental stage of *C. elegans* can be readily determined by microscopic examination. The number of worms on a plate or liquid culture can be counted and viewed under a wild M3 stereo microscope, under 3 different magnifications (6.4X, 16X and 40 X) .

Section 2.2.5 growth of *C. elegans* in liquid medium

liquid cultures are often used to grow large quantities of *C. elegans* for biochemical, nutritional, toxicological assays, or to obtain synchronous cultures. There are many benefits of growing worms in liquid culture. A good example for this is that the liquid culture is constantly mixed, so the worms are exposed to identical conditions of pH, food, temperature and oxygen concentrations.

This kind of culture is generally grown in S-medium with *E. coli* OP50 added as a food source. Overnight cultures of *E. coli* OP50 were grown in LB medium to create concentrated pellets of bacteria. These pellets can be stored at 4°C for several weeks or in a -80°C freezer indefinitely. The amount of bacteria and the number of adults depend on the strain of worm used, and the length of time the worms are grown. These conditions and the amount of bacteria provided are extremely important for the number of adults input as well. Every liquid culture, however, grows just one generation of worms before the worms need to be harvested. The major reason for this is that when growing worms for more than one generation, there

will be a huge number of animals, which can very often lead to dauer production, despite the presence of food.

Section 2.2.5.1 Liquid medium

Day 1

- 1) Single colonies of OP50 were incubated with a 5 ml liquid LB medium and incubated (240 rpm, 37 C) overnight.

Day 2

- 2) This was seeded to 500ml of liquid LB medium into one a litre flask which was incubated overnight (240 rpm, 37 C).
- 3) Then 500ml of liquid LB medium (Bacteria) was decanted into two 500 ml centrifuge bottles and spun at 4000 rpm for 10 min.
- 4) S.medium was made up as describe in the Materials section.
- 5) A concentrated *E. coli* OP50 pellet was added to Smedium in a fresh 1-litre flask. A measurement of the optical density (OD) was conducted using a Spectrophotometer.
- 6) 4 large plates of starved worms and uncontaminated N2 or *lat-1(ok1465)* worms were washed (just cleared of bacteria). They were washed twice with S Medium or M9 buffer into 15 ml centrifuge tubes and spun for 2 min at 1500 rpm.
- 7) They were transferred into liquid culture (10 ml into a 50 ml tube or 400ml into a one litre autoclaved flask).
- 8) The flask or 50 ml centrifuge tube cultures were incubated at 20°C and 240 rpm on an orbital shaker so that liquid cultures were well oxygenated.
- 9) A 1 mL sample was taken every day to check growth and the optical density of food and whether a duers were forming. The presence of duers may be caused by either too much or too little food or of the culture is too dense. Too much food can suffocate the worms

from lack of oxygen. However, the food supply should be checked under the microscope. The sample should not be visibly cloudy.

- 10) If the food supply was depleted, more *E. coli* OP50 suspended in S Medium was added otherwise eggs hatch inside hermaphrodites, and dauer larvae may be produced.
- 11) After synchronous growth the worms should be gravid adults and ready to harvest. This is usually on the 4th or 5th day. The adult worms grown in liquid culture are usually longer and thinner than worms harvested on Petri plates, and tend to hold their eggs.

Section 2.2.5.2 Harvesting worms to isolate embryos for starting a synchronized culture.

After growing a large number of worms in liquid culture, the majority of the worms should be adults with embryos. This method is preferable, because normal bleaching as used for plate-grown worms does not work well with liquid culture.

- 1) Before worm harvesting was started, the number of adults in every flask was checked by mixing the liquid culture then transferring 50 µl of the liquid culture to a slide and viewing under a microscope. If there were a lot of gravid adults, the worms were ready for bleaching.
- 2) The flask (400ml of liquid culture) was put on ice for 30 minutes and the worms were to left settle. If there were more flasks (each with 400ml of liquid culture), two flasks were collected together into a one liter flask which was put on ice for one hour.
- 3) The medium was aspirated off until approximately 100 ml was left, then transferred to four 50 ml falcon tubes. These tubes were put on ice for 20 min and the remaining media was removed until only 10ml remained.
- 4) Ice cold of M9 was added to 10 ml of worms and bacteria.
- 5) The 50 ml falcon tubes were spun at 1200 rpm for 2 mins.
- 6) The medium was aspirated off until 10 ml remained.

- 7) Ice cold M9 was added to 50 ml and spun down again.
- 8) After aspirating off the M9, the worm pellet was resuspended in 25 ml of ice cold M9 and 25 ml of ice cold 60% (w/v) sucrose (60 g of sucrose dissolved in 10 ml of distilled water) was added. Worms were cleaned from debris by sucrose floatation. This was an essential step before isolating embryos.
- 9) The contents of each tube were mixed and spun immediately at 2800 rpm for 5-6 minutes(a partner tube of 25ml M9 buffer and 25ml of 60% (w/v) sucrose must be spun as balance).
- 10) The adult worms should be visible as a brown film on top of the tube, whereas the bacterial debris has pelleted.
- 11) The worms were collected (the top most 30 ml of the media before getting to the pellet) with a 25 ml pipette or by a cutoff pipette with a wide tip. This was transferred to a new 50 ml tube.
- 12) The 30 ml of the medium plus worms (worms) was divided into two 50 ml tubes.
- 13) Ice cold M9 was added to bring the volume to 50 ml in the both tubes and was spun at 1200 rpm for 5 min. If the sample was not diluted with M9 buffer sufficiently, the worms will not pellet.
- 14) The pellet of worms was washed twice more with M9 buffer and the worms were observed under a dissecting microscope.
- 15) 25 ml ice cold 0.1M NaCl was added to the worm pellet.
- 16) The worms were left for 5 min to settle and the supernatant was aspirated.
- 17) Ice cold 0.1M NaCl up to a volume of 30 ml was added.
- 18) 5 ml 5M NaOH (2.5 ml of 10 M NaOH and 2.5 ml of distilled water) was mixed with 10 ml bleach in a 15 ml tube.

- 19) The NaOH/bleach solution was added immediately to the 30 ml worm suspension in (0.1M NaCl).
- 20) The tube was vortexed for 5 seconds and left to stand at room temperature for 2 minutes then vortexed again.
- 21) Step 20 was repeated four times for a total bleaching time of 10-15 minutes. The progress was examined by transferring samples under the dissecting microscope. Bleaching was stopped when only embryos remained.
- 22) The worms were then centrifuged at 1200rpm for 1 minute and the supernatant was aspirated off.
- 23) Ice-cold of K medium was added to a total volume of 50 mL.
- 24) The tube was centrifuged at 1200rpm for 2 minutes and the supernatant removed.
- 25) Steps 23 and 24 were repeated twice to make sure all the eggs were clean from the bleaching solution.
- 26) Finally, the worm pellet mixed with K medium was transferred to six well plates, adding ~ 1ml per well, because there were lots of eggs present. This means that the L1 worms get oxygen more easily.
- 27) The six well plates were incubated at 15°C for 18-20 hours to allow the embryos to hatch.
- 28) L1 worms were transferred to liquid culture.

Section 2.2.6 Mutagenesis

Section 2.2.6.1 EMS method

Ethyl methanesulfonate (EMS) is stored at room temperature, and must be handled in a fume hood with gloves. 1 ml M9 and 20µl of EMS solution were placed in a 15 ml tube and mixed until no longer cloudy. The mixture was added to 3ml of M9 buffer containing worms

to get 50mM EMS(20 μ l of EMS in 4 ml M9 = 50mM). After finishing the mutagenesis process, all pipets/tubes contaminated with EMS and the supernatant of washing worms with EMS were inactivated by mixing them with an equal volume of sodium thiosulfate (0.1M NaOH, 20% w/v Na₂S₂O₃) for 24 hours.

Section 2.2.6.2 Culturing *lat-1(ok1465)* worms for mutagenesis

Two methods were used to preform the mutagenesis for *lat-1(ok1465)* worms.

Section 2.2.6.2.1 First method

- 1) Grow a large number of *lat-1(ok1465)* worms.
- 2) The large number of gravid adults (*lat-1*) from a large_scale liquid culture (10 flasks) was treated with egg isolation buffer to harvest the eggs as described in section 2.2.5.2. To make sure that all the worms were at L1 stage next day, the eggs with K Medium were incubated at 15 degree. Because there was no food present, all the hatching eggs were at the L1 stage.
- 3) Next day, the L1 worms were transferred to liquid culture and incubated at 20°C in three flasks.
- 4) After three days of growth at 20°C, each flask (400ml of liquid) was put onto ice for 30 minutes and the supernatant was removed.
- 5) The worm pellet (L4) was divided into four 15ml centrifuge tubes and were centrifuged at 1200 rpm for 30 sec. This procedure was done once or twice to remove excess bacteria before the mutagenic treatment.
- 6) The worm pellet was resuspended in 3 ml of M9.
- 7) The L4 worms were mutagenised using EMS as outlined in figure 2.1,,using 50mM EMS as described in section 2.2.6.1.
- 8) The 15ml centrifuge tubes were put into the incubator at 20°C for 4-5 hours.

- 9) After treatment, worms were washed twice by M9 buffer to remove the EMS solution and transferred into liquid culture.
- 10) Mutagenised L4 *Lat-1(ok1465)* mutagenesis worms were left growing in liquid medium for two days until gravid then the number of adults was counted (P0 generation worms).
- 11) The gravid adults were bleached to isolate the eggs, which contain the F1 progeny of the mutagenised animals.
- 12) F1 worms were grown at 20°C for 4 days until they became adults.
- 13) F1 adults were bleached to isolation the eggs, which represent the F2 generation.
- 14) F2 worms were transferred at the L1 stage to liquid culture and grown at 20°C for 3 days, until they reached the L4 stage.
- 15) L4 worms were screened as described in section 2.2.6.3.

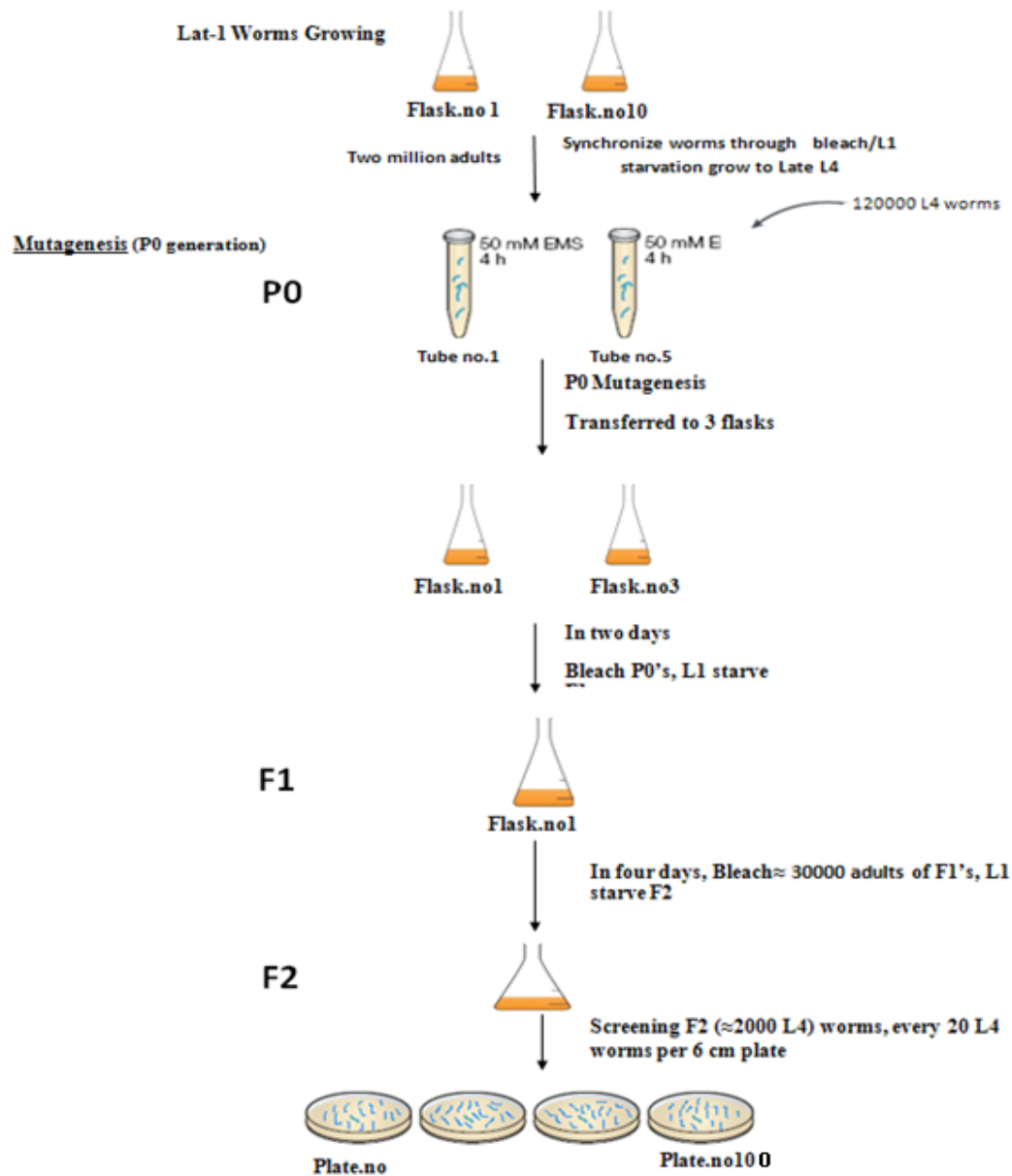


Figure 2.2.1 The mutagenesis method

This figure outlines the mutagenesis of the *lat-1(ok1465)* worm. It can be seen from the figure that a large number of *lat-* worms were bleached to get the P0 generation. P0 generation was mutagenesed by EMS, when they reach the L4 stage. Then P0 gravid worms were bleached to get first generation (F1). After four days, the gravid adults were bleached to get the second generation (F2). The F2 progeny are inspected for the phenotype of interest. Therefore, F2 mutants are screened by transferring the 20 of L4 worms to a fresh plate to evaluate if the characterbreeds true.

Section 2.2.6.2.2 method two

- 1) The *lat-1(ok1465)* worms were grown in one flask.
- 2) Gravid adults (*lat-1*) were washed off from liquid culture and were treated with egg isolation buffer to harvest the eggs as described in section 2.2.5.2.
- 3) When the eggs hatched, all the L1 worms were transferred to liquid culture.
- 4) After three days of growth at 20°C, the flask (400ml of liquid) was put onto ice for 30 minutes and the supernatant was removed.
- 5) The worm pellet (mostly L4) was divided among four 15ml centrifuge tubes. ~10ml of M9 buffer was added to each tube, and they were centrifuged at 1200 rpm for 30 sec. This procedure was done once or twice to remove excess bacteria before the mutagenesis treatment.
- 6) Worm pellet were resuspended in 3 ml of M9 buffer per tube.
- 7) The L4 worms were mutagenised using 50mM EMS, as described in section 2.2.6.
- 8) Mutagenesised L4 worms were transferred to ten 4.5 cm plates (ten groups).
- 9) After two days all the P0 adults from every plate were washed off while the eggs remained stuck to the bacteria.
- 10) The following day the plates were checked to see if the eggs had hatched (F1 generation worms)
- 11) After four days all the F1 adults from every plate were washed off leaving the eggs stuck to the bacteria.
- 12) After a further three days, there were L4 worms in every plate, which represent the F2 generation.
- 13) The L4 worms on every plate were screened as described in section 2.2.6.3.

Section 2.2.6.3 Screening F2 mutants

Random mutagenesis of *lat-1(ok1465)* animals was carried out. A mutagenised population can be screened for visible mutants, but recessive mutations will only be revealed in the F2 population.

- 1) When the F2 worms reached the L4 stage, the F2 adults were counted.
- 2) 5-10 μ l of worms were transferred to give 20 worms per aliquot.
- 3) Each aliquot of 20 L4 worms was transferred to a small (3 cm) plate and checked under a microscope to make sure there were just ~ 20 L4 worms.
- 4) After five days, the self-progeny were screened on every plate, and the number of offspring was counted.
- 5) Candidate plates were selected where progeny number were larger than expected, and the brood size of individual worms was determined.

Section 2.2.7 Polymerase chain reaction (PCR)

- 1) A single worm (or a few worms) was directly transferred into a 500 μ l PCR tube with 10 μ l of lysis buffer.
- 2) The worm was then frozen at -80 for 1 hour. The freeze/thaw helps to lyse the worm in order to isolate the genomic DNA.
- 3) The 500 μ l PCR tube with worms and 10 μ l of lysis buffer was then incubated for 6 hours at 65°C then 1 hour at 95°C. The proteinaseK was active at 65°C but inactivate when heated to 95 °C. This is essential for proteinaseK which can cleave the polymerase during the PCR reaction.
- 4) The reaction mixture consist of 2 μ l of the DNA solution, 1 μ l of each forward and raverse primer 10pmole primers and 26 μ l of PCR master mix which added to each

PCR reaction tube. PCR hood stops environmental contamination. Using clean tips and equipment prevents cross contamination.

- 5) The following conditions of the PCR reaction were carried out in a Progene thermal cycler.
 - a) 94°C for 30 seconds - Denaturing
 - b) 54°C for 30 Seconds - Annealing
 - c) 72°C for 1 minute – Elongation
 - d) This was done for 35 cycles.
 - e) The essential step before starting previous conditions was that cycling the PCR reaction was heated for 2 minutes at 94°C. This was required for the heat activation of DNA polymerase.
- 6) The PCR reaction was run on a 12% polyacrylamide gel electrophoresis (PAGE) alongside a negative control and a DNA ladder as a marker. This was to confirm the amplification of the PCR product

3. Results

Section 3.1 Brood size analysis

This experiment was performed to investigate the difference in brood size between wild type N2 worms and *lat-1(ok1465)* worms. Wild-type hermaphrodites were used as a positive control. Brood size was determined as described in section 2.2,4.1. Each hermaphrodite offspring was counted individually at the adult stage and the number of offspring hermaphrodite was expressed as the mean \pm standard deviation. The brood size for N2 worms was 295 ± 36 worms (mean \pm standard deviation, $n=10$). The brood size assay for *lat-1(ok1465)* worms was 4 ± 4 ($n=10$). Data were analyzed by t-test and there was a significant difference ($P=0.0001$) between N2 brood size and *lat-1(ok1465)* brood size.

Section 3.1.1 Brood size analysis of *lat-1(ok1465)* worms and Hawaiian background

The aim of this experiment is to see whether the Hawaiian background affected the brood size of *lat-1(ok1465)* worms. The *ok1465* allele had been bred onto the Hawaiian (CB4856) background for six generations (C. Budd, M.Res. thesis, 2008). Brood size was determined as described in section 2.2.4.1. Each hermaphrodite offspring was counted individually at the adult stage and the number of offspring hermaphrodite was expressed as the mean \pm standard deviation. It was found that the brood size of *lat-1(ok1465)* worms on the Hawaiian background is 6 ± 4 . There was no significant difference between the brood size of N2 *lat-1(ok1465)* worms on the N2 and Hawaiian backgrounds ($P=0.0642$).

Section 3.2 Yield of worms after bleaching

Each *lat-1(ok1465)* worm gives approximately four offspring (adults) during three days. It was originally envisaged that the mutagenesis method would require multiple rounds of bleaching to synchronise the worms, and to deal with the large volumes of liquid culture of *lat-1(ok1465)* worms. This experiment was performed to determine the number of *lat-1(ok1465)* adults in the first generation (F1) after bleaching a particular number of adults in the parental generation (P0). This was compared with N2 worms which as the positive control. This experiment was performed by bleaching a known number of adults in the parental generation (P0). Eggs were transferred to 6-well plates and incubated at 15 °C overnight in the absence of food to make sure all progeny were at the L1s stage. The next day, the number of L1 per 100 P0 worms was counted. After three days of incubation at 20°C, the number of adults in the first generation (F1) was counted per 100 P0 worms. At this stage only gravid adults were counted. This experiment was repeated five times for N2 (table 3.1) and for *lat-1(ok1465)* worms (Table 3.2).

P0	L1/ per100 P0	F1 adultsper 100 P0
4000	425	425
3500	428	428
3500	462	462
3000	350	350
2000	400	400
Mean \pm standard deviation	413 \pm 41	413 \pm 41

Table 3.1 Recovery of N2 worms after bleaching

N2 worms were bleached and the number of L1 was counted the next day. After three days the number of adults F1 worms was counted. F1 worm number are expressed as a ratio to 100 input P0 worms.

Table 3.1 shows the number of F1 wild type L1 larval and adults obtained from bleaching 100 P0 adults - around 4. The percentage of wild type L1 which died before reaching adulthood was 0%.

P0	L1/ per100 P0	F1 adults per100 P0
4000	250	18
3500	250	16
3500	214	14
3000	111	11
2000	150	15
Mean \pm standard deviation	195 \pm 62	14 \pm 2

Table 3.2 Recovery of *lat-1(ok1465)* worms after bleaching .

Lat-1(ok1465) worms were bleached and the number of L1 was counted the next day. After three days the number of adults F1 worms was counted. F1 worm numbers are expressed as a ratio to 100 input P0 worms.

Table 3.2 shows the number of F1 adults which were obtained from bleaching a particular number of *lat-1(ok1465)* adults (P0). Table 3.2 shows that 195 \pm 62 L1 worms can be produced from bleaching 100 (P0) adults and 14 \pm 2 adults (F1) survived from these L1 worms. Therefore around 93 % of L1 worms died before reaching adulthood. According to this table, the efficiency of bleaching for *lat-1(ok1465)* worms was 0.14 offspring per input adult worm. Therefore, more of the *Lat-1(ok1465)* worms died during early larval stages before they reach adulthood. This shows that a large number of *lat-1(ok1465)* worms are required to obtain synchronised *lat-1(ok1465)* worms for mutagenesis.

Section 3.3 The number of *Lat-1(ok1465)* worms required for mutagenesis

From the previous section, it is possible to calculate the number of starting adults which should be used to get a particular number of second generation offspring. This calculation was performed to determine the number of *lat-1(ok1465)* required for undertaking a mutagenesis screen (parental generation offspring) assuming bleaching at each generation and screening of large number of worms in the second generation.

Statistics based on the data from table 3.2 were used to calculate the number of starting adults that should be used to get the necessary number of L4 worms (P0) for mutagenesis. In addition, the number of first generation (F1) worms can be seen from table 3.3.

start	P0 (L4)	F1	F2
171500	24500	3500	500
343000	49000	7000	1000
686000	98000	14000	2000
6860000	980000	140000	20000

Table 3.3 The number of *lat-1(ok1465)* for mutagenesis

Equation 3.1 the calculation number of *lat-1(ok1465)* for mutagenesis

1) Start= $F2 (100/14)^3$

2) P0= $F2 (100/14)^2$

3) F1= $F2 (100/14)$

Equation 3.1 allows estimation of the number of adults which are required to screen 500, 1000, 2000 or more worms at second generation (F2).

Table 3.3 shows the number of *lat-1(ok1465)* worms which must be cultured to yield a particular number of second generation worms (F2). For example, 171500 *lat-1(ok1465)* adults worms should be bleached to mutagenise 24500 L4 parental generation offspring (P0), which lead to 3500 adults (F1) after bleaching. 500 *lat-1(ok1465)* L4 worms (F2) would then be screened. It can also be seen from this table that screening 20000 *lat-1(ok1465)* L4 worms at F2 requires approximately 6.9×10^6 adults. It was conclude that large scale culture of worms is required so that $\sim 10^7$ worms can be grown.

Section 3.4 Growing worms in liquid culture

Table 3.2 shows that a large number of *lat-1(ok1465)* worms must be grown for the mutagenesis method, requiring that *C. elegans* should be grown in liquid culture. Therefore, an experiment was conducted to see how N2 and *lat-1(ok1465)* worms grow in liquid culture.

Section 3.4.1 Growth of N2 worms in liquid culture

This experiment needs to be calibrated to get the optimal bacterial concentration which is measured using optical density (OD) at a wavelength of 600 nm. Also the optimal temperature of growth, the speed of shaking (rpm) and the initial number of worms needed at the start is required. The aim of this experiment is to find the maximum number of worms which can be grown in 10 ml of liquid culture.

The wild type worm (N2) was grown as a positive control. N2 worms were tested at different bacterial concentration, i.e. optical densities of 2.2, 2.0, 1.7, 1.5, and 1.0. Bacteria were grown at 37C° and 200 rpm. Then, S medium was added to the pellet of bacteria, until the optical density at 600 nm reached 2.2, 2.0, 1.7, 1.5 and 1.0. It was found that adding 250 ml of fresh S medium to 400 ml of an overnight culture gave an OD of 1.5 at 600 nm. Approximately 1000 worms were tested at each O.D as shown in figure 3.1. Then the number of N2 worms (adults) was counted daily. This was done by taking 30 µl of liquid culture three times and counting the worms to get the average and standard deviation.

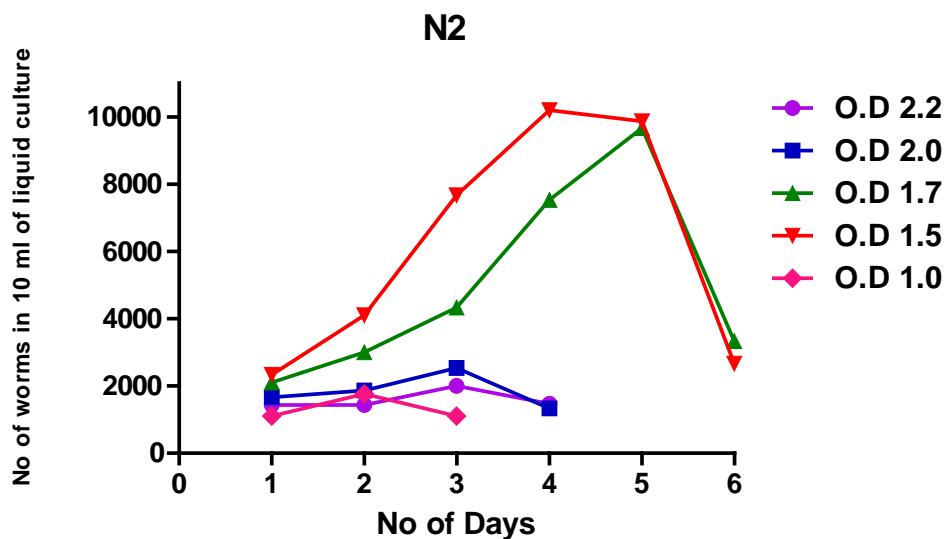


Figure 3.1 The growth of N2 worms at different bacterial concentration

The vertical axis shows the number of adult worms in 10 ml of liquid culture and the horizontal axis indicates the number of days. 1000 N2 worms were transferred to each 10 ml tube of liquid culture and incubated at 20 C°. Each 10ml of liquid culture had a different O.D (bacterial concentration). The number of worms was counted daily by taking 30 µl from every tube. This was repeated three times and the average was taken.

It can be seen from figure 3.1 that the number of worms increased slightly on the first and second days at an O.D. of 2.2 and 2.0 at 600 nm. On the fourth day both of them slightly decreased. This might mean that the high bacterial concentration might be too dense, as the worms can suffocate from lack of oxygen. In agreement with this, the hermaphrodites were small and produced few eggs with many of them dying. On the other hand, the number of worms dramatically increased at an OD of 1.7 and 1.5 at 600 nm. The number of worms reached a peak on day five and had approximately 1000 hermaphrodites/ml and then the worms dramatically decreased on the sixth day as shown in figure 3.1.

Figure 3.1 shows that optimal bacterial OD at 600 nm is between 1.5 and 1.7. In addition, approximately 10000 worms can be grown into 10ml of liquid culture. A count was taken after 6 days and was checked on day four and five to make sure that there was enough food and the worm density was not too high. If the food was almost finished and worm

density was high, the worms were harvested at once. After seven days, the liquid culture was clear of food. Eggs hatched inside the hermaphrodite were seen by day seven.

Section 3.4.2 Growth of N2 worms in liquid culture with bacteria at 1.7 and 1.5 (OD_{600nm})

This experiment was performed to confirm the number of worms which can be grown in 10 ml of liquid culture at 1.7 and 1.5 OD at 600nm. Four tubes were counted three times a day to get the average and standard derivation. The total number for each OD (1.5 and 1.7) is shown in figure 3.2.

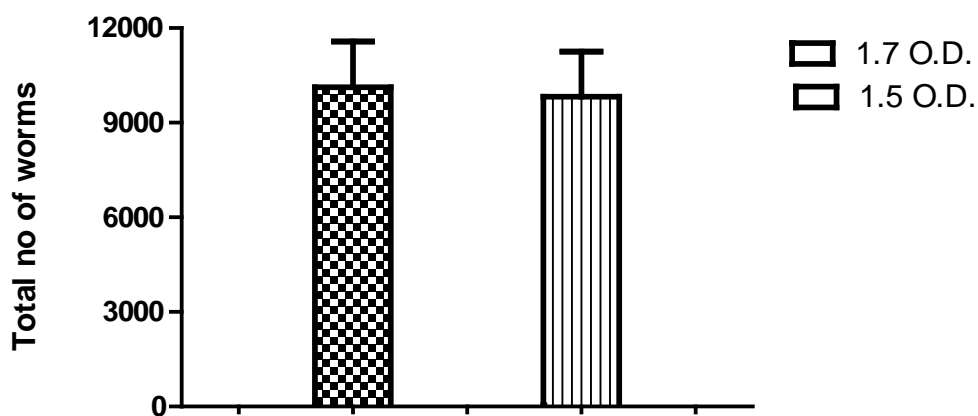


Figure 3.2 Growth of N2 worms in liquid culture

The vertical axis shows the total number of worms in 10 ml of liquid culture and horizontal axis indicates the optical density. 1000 worms were transferred to 10 ml of liquid culture which had either an O.D of 1.5 or 1.7 *and incubated at 20 C°*. This was repeated four times for every O.D (1.7 and 1.5). The number of worms from each tube was counted daily, until the number of worm decreased. Then, the total number of worms was counted to get the average and standard deviation ($n=4\pm SD$).

From this graph, it can seen that on day 6 approximately 10,000 hermaphrodites can be grown in 10 ml of liquid culture at an OD of 1.7 or 1.5 at 600 nm.

Section 3.4.3 use of a large inoculum of N2 worms

This experiment was performed to monitor see the growthwhen starting with a large number of worms, using 15000 worms in 10 ml of liquid culture.

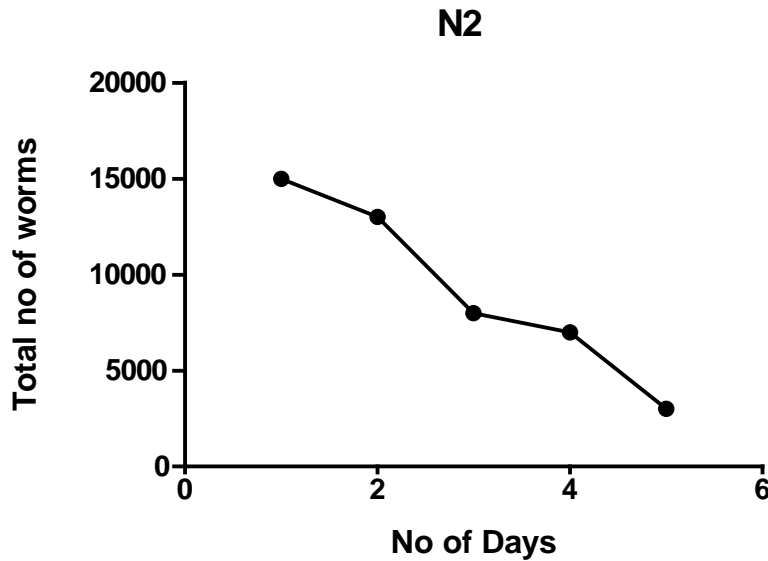


Figure 3.3 Use of a large inoculum of N2 worms in liquid culture

The vertical axis shows the total number of worms in 10 ml of liquid culture and horizontal axis indicates the number of days. This was started with 15000 N2 worms and incubated at 20 C°. The number of worms was counted daily in 30 µl aliquots. This was repeated three times to get the average of the number of worms n=3.

From figure 3.3 it can be seen that the number of worms gradually decreased over 5 days. After four days, eggs hatched inside the adults' body. This shows that 15000 worms in 10 ml of liquid culture were too many. Therefore, starting with a small number was better than starting with a large number.

Section 3.4.4 growth of *lat-1(ok1465)* worms

This experiment was performed to see how *lat-1(ok1465)* worms grow in liquid culture. *Lat-1(ok1465)* worms were tested using the best OD (600nm) as found in the N2 experiment, starting with either 500 or 1000 worms. The growth kinetics of *lat-1(ok1465)* worms in the liquid culture were determined by counting the number of worms every three days until numbers decreased. It was found that *lat-1(ok1465)* worms grow more slowly than N2 and required extra time to yield enough worms, taking about 10 days to reach 10,000 worms as shown in figure 3.4.

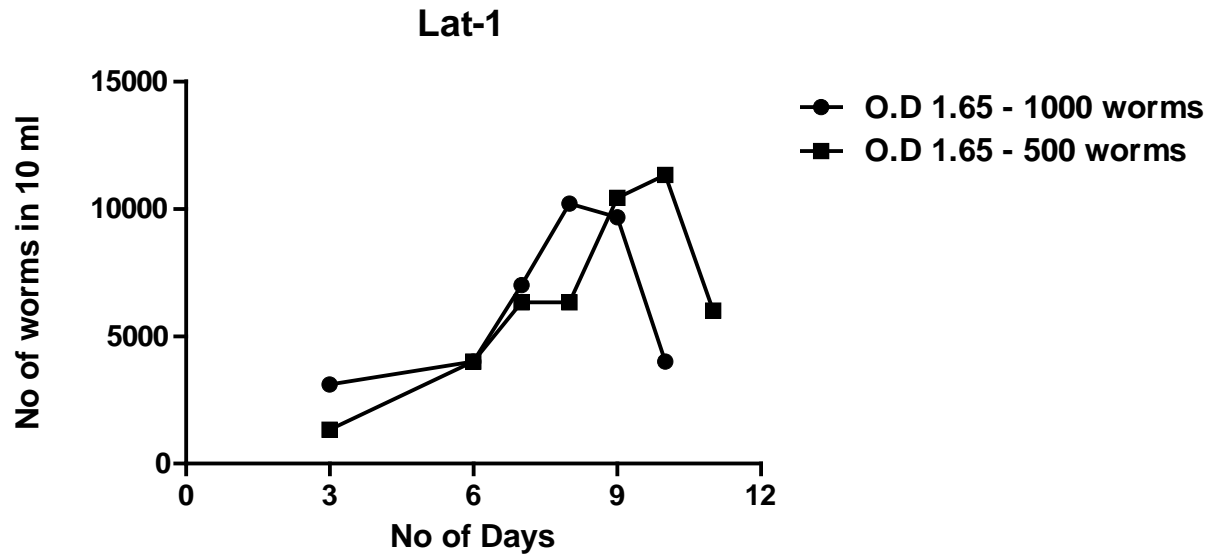


Figure 3.4 The growth of *lat-1(ok1465)* worms at O.D. of 1.65 at 600 nm.

The vertical axis shows the total number of worms in 10 ml of liquid culture and horizontal axis indicates the number of days. Two different starting numbers were used, 500 worms and 1000 worms (adults) both with an O.D 1.6 which incubated at 20 C°. The number of worms was counted every three days until the number of worms decreased. This was done by taking three 30 μ l aliquots of liquid culture and counting the worms in each (n=3).

Figure 3.4 shows that the number of *lat-1(ok1465)* worm reached a peak after ~ 9 days, when starting with 1000 worms and 11 days, when starting with 500 worms. The maximum number of worms was approximately 10000 worms. Therefore, the *lat-1(ok1465)* worms can be grown in liquid culture.

It was concluded that N2 worms and *lat-1(ok1465)* can be grown in liquid medium as shown in figures 3.1 and 3.4, using bacteria at an OD of 1.5-1.7 at 600 nm (shaking at 20C° on a platform shaker at ~200 rpm). A small inoculum size was better than large, as seen when comparing figures 3.1 and 3.3. Moreover, approximately 1000 worms can be grown in 1ml of liquid culture.

It can be seen from figure 3.1 and 3.4 that growing *lat-1(ok1465)* worms required longer (~10days) than for N2 worms (5 days) in liquid culture, starting with similar small numbers of worms.

Section 3.4.5 Growing large number of *lat-1(ok1465)* worm

This experiment was performed to grow *lat-1(ok1465)* worms in a large amount of liquid culture to determine the time course and the yield of worms.

The experiment was started with ten flasks. Each flask had 400ml of liquid culture with an OD of 1.65 at 600 nm. The worms were grown on plates for three days and transferred to a litre flask (400 ml of liquid culture). After three days, the worms were washed down and transferred to ten flasks. The growth of worms in the flask was compared with the plates.

Initially, a large number of worms (60000 worms per flask) was used. Since the density of worms was high, consequently the bacteria were almost depleted in the flask. Thus bacteria were added as appropriate to avoid worms dying or reaching dauer stage. The disadvantage of adding more bacteria is that bleaching worms was found to be difficult. It was decided to use a smaller number of worms (15000) per flask.

When using a small starting number, there is no need to add bacteria every day. Bleaching could be performed easily because no bacteria were found at the end of 10 days.

Lat-1(ok1465) worms were grown in ten flasks, each with around 15000 adults in 400ml. After seven days, the number of worms in triplicate 40µl aliquots was counted (n=3). From this, it was found that around 2.1×10^5 *lat-1(ok1465)* worms can be grown in a flask at an OD of 1.65 at 600 nm after five to six days of growth.

Growing worms in ten flasks was compared against growth in ten 9 cm plates. It was found that all the flasks had large number of worms, but some plates had no worms at all, in

spite of growing them under in the same conditions. Moreover, the bleaching of one flask gave more eggs than bleaching ten 9 cm plates.

Section 3.5 Mutagenesis

The *lat-1(ok1465)* worm has a deletion in the *lat-1(ok1465)* gene, as described in section 1.2.4. Each *lat-1(ok1465)* worm gives only 4 ± 4 adult offspring. The mutagenesis strategy was performed to see if mutants with an increase in brood size can be obtained. The reason for doing this is that these mutants may identify genes that control *lat-1(ok1465)* mediated lethality, and hence may explain how the *lat-1(ok1465)* signalling pathway works.

Section 3.5.1 Size of the mutagenesis screen

A key aspect is to estimate the number of second generation (F2) mutagenized genomes of *lat-1(ok1465)* worms which should be screened.

After mutagenesis, each P0 generation animal contributes two copies of each gene. Therefore, every 1000 N2 F1 animal has 2000 mutagenised copies of the genes. The *C. elegans* genome has about 20,000 genes. 50 mM ethyl methanesulfonate (EMS) induces loss-of-function mutations in worms at an average frequency of one null mutation per 2,000 copies of the genes (Brenner, 1974). This mutation frequency is an average and does not guarantee the target gene will be detected. Therefore, the binomial distribution was used to obtain the probability of not getting a specific gene, when screening particular number of genomes as shown in table 3.4.

Number of genomes	F1	F2	% of genes with no mutation
2000	1000	4000	24.2
4000	2000	8000	10.4
6000	3000	12000	5.1
8000	4000	16000	2.7

Table 3.4: The number of genomes required for screening

This table shows the number of first and second generation worms for N2 that would be needed to be screened particular number of genome. In addition, a binomial distribution was used to calculate the percentage of not getting a mutation in any give gene.

From table 3.4 it can be observed that screening 4000 genomes in the wild type animal, 8000 F2 from 2000 F1 animals should be screened. This would mean two null mutations per gene on average. Approximately 16000 F2 worms should be screened to obtain 97% coverage of the genome.

Section 3.5.2 The number of F2 animals screened on a single plate

A typical genetic screen in *C. elegans* isolates recessive mutations (loss-of-function alleles) in F2 generation worms. A resistant mutant in *lat-1(ok1465)* worms would produce up to 300+ viable offspring, more offspring than produced by *lat-1(ok1465)* worms (4 ± 4). Therefore, the F2 generation should be screened by picking one or more worms in small plates to measure brood size in mutagenesis *lat-1(ok1465)* worms.

Section 3.5.1 showed that ~ 16,000 F2 *lat-1(ok1465)* should be screened to identify a mutant affecting a gene of choice. An optimal screening strategy should be defined based on the ability to detect an increased brood size in F2 worms. 4 options were used to investigate the optimal screening strategy for this number of worms (table 3.5).

The number of worms (F2) per plate	Number of F3 offspring (No mutant)	Number of F3 offspring (1 mutant worm with brood size of 300)	The number of plate to screen 20,000 worms
1	4	304	20,000
2000	8000	8300	10
10	40	340	2000
20	80	380	1000

Table 3.5: Screening the number of worms per plate

The number of worms in the second mutagenesis generation should be screened to identify the gene that leads to increase in the brood size. This table shows four options to screen 20,000 F2 generation worms.

From the table 3.5 can be seen that screening F2 of 2×10^4 worms individually would give maximum ability to detect a brood size mutant, but it would be time consuming to screen 2×10^4 plates. Therefore, more F2 worms should be screened together in each small 3cm plate. Table 3.5 shows that one adult *lat-1(ok1465)* worm gives 4 offspring, so 2000 adults are expected to give 8000 offspring. If one of the 2000 worms had an increased brood size, 8300 offspring are expected. It would not be possible to see the difference between 8000 and 8300 worms. Therefore, screening 2000 worms on an individual plate is impractical. The third option is that screening 10 worms on an individual plate gives 40 adults and the difference between 40 and 340 is very large, but the difficulty is transferring 20,000 worms on to 2000 plates. From this table it can be concluded that transferring and screening 20 worms on each individual plate is still big enough to tell the difference by counting between 80 and 380. Therefore, screening 2×10^4 F2 worms at 20 worms per plate should theoretically be a practicable method for isolating mutants.

This scheme was practically tested to determine if worm numbers were sufficiently consistent for large-scale screening. *lat-1(ok1465)* worms were bleached. Then, the eggs were transferred on to a 6 well plates overnight. The L1 worms were transferred into liquid culture at 20C° for three days. When the worms reach L4 stage, groups of 20 worms were transferred onto a small plate. Initially, the concentration of worms in solution was

determined and an appropriate volume was transferred to a plate without looking under the microscope. After four days, the offspring was counted to get the average \pm standard deviation $n=10$.

It was found that the plates had 128 ± 55 worms. This was unacceptable, because the number of offspring was too high. Therefore, the experiment was repeated, but this time plates were visually checked every time under the microscope to make sure the number of worms was around 20 worms. Unmutagenized control animals had an average of 74 ± 14 offspring per plate.

Section 3.5.3 Mutagenesis of *lat-1(ok1465)* worms

Mutagenesis can induce mutations which affect vulva development and an easily seen phenotype is multi-vulva. EMS-treatment was examined to see if the mutagenesis protocol worked in *lat-1(ok1465)* worms.

Lat-1(ok1465) worms were mutagenized with 50mM EMS as described in section 2.2.6. When second generation mutagenesised worms (F2) reached adult stage, they were screened for the multiple vulva phenotype.

Thirty independent plates from a total of 800 plates screened had worms with the multi-vulva phenotype as shown in figure 3.5. Therefore, the mutagenesis has yielded a high number of mutant worms.

800 plates were screened which had 20 worms in each plate. From this, we found that 30 plates had multi-vulva phenotype that give the incidence, 30 mutants per 16,000 F1, or 30 mutants per 8000 copies of the genome at F1. This was more than expected, but the actual expected number is 4 null mutations. According to a previous study, 37 mutants per 17,000

copies of the genome with various vulva developmental defects were found (Antoshechkin and Han, 1998).

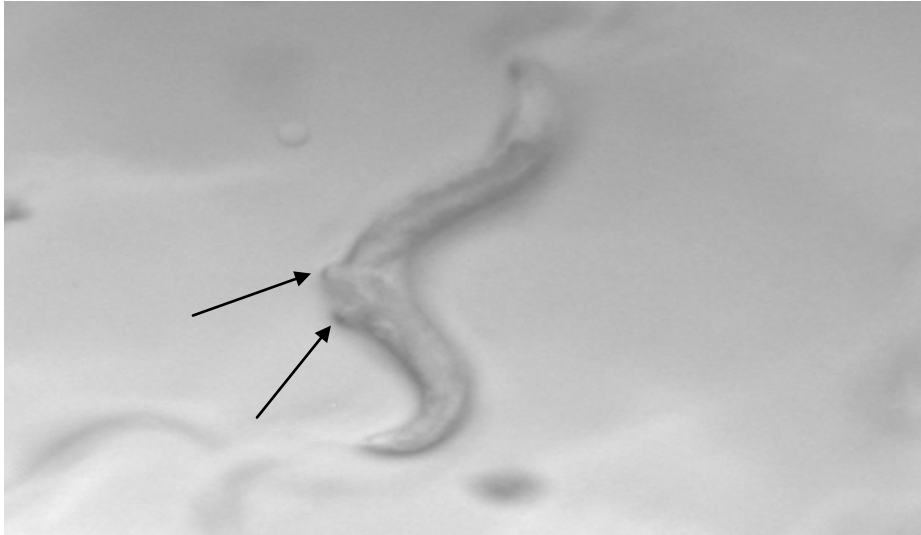


Figure 3.5: Multi-vulva phenotype

This picture shows a single worm with multi-vulva phenotype. *Lat-1(ok1465)* worms were treated with 50 mM EMS and the worms were screened. We found multi-vulva development in the F2 generation worms. The arrows show the position of vulva.

Section 3.5.4 Screening plates for mutants with an increase brood size

The aim for this experiment is to identify specific mutants of *lat-1(ok1465)* worms with an increase brood size.

Lat-1(ok1465) worms were mutagenized and the P0 generation worms and the F1 generation worms were bleached to obtain a synchronized culture. The F2 generation was screened by transferring 20 L4 worms to each small plate (3cm). After four days the number of offspring was counted. The first 10 plate's offspring was counted to get the average and standard deviation. Other plates were screening by comparing with the number of offspring in previous 10 plates.

300 plates were screened to identify whether any one of the plates had a large number of offspring. 10 plates were found to have approximately 54 ± 27 . It was found that all 300 plates had approximately ≤ 80 worms. Therefore, in these 300 plates there were no *lat-1(ok1465)* worms with an increased brood size.

The bleaching of P0 and F1 worms in the previous experiment produced very low numbers of offspring. Therefore, in the next screen, *lat-1(ok1465)* worms were left in the plate. The worms were divided into ten groups at F1 stage. If two or more resistant worms were found in one group, they might have the same mutation.

10000 L4 *lat-1(ok1465)* worms were mutagenized as described in section 2.2.6.2.2. Then, 1000 mutagenised worms (P0) were transferred to 4.5 cm plates (ten groups) with high peptone. After two days, the adults worms were washed off and the eggs remained, stuck to the bacteria. After four days, the F1 adults were washed off and the F2 eggs were left adhering to the bacteria. After, three days the F2 generation worms were screened by transferring 20 L4 worms to each small plate (3cm).

500 plates were screened to identify any plate that had large numbers of offspring. There were two plates which had a large number offspring. These plates had approximately 130 and 212 worms respectively. This number of worms was found to be more than unmutagenized control animals, which had an average of 74 ± 14 worms. One plate was from group three and the other plate was from group six.

Section 3.5.4.1 Brood size of two candidate mutant lines

This experiment was preformed to examine the two plates, which were found from screening as described in section 3.5.4.

20 L4 worms from each plate were selfed onto different plates. After four days the offspring of each plate was counted.

It was found that two worms from group six produce high number of offspring as shown in figure 3.8. Therefore, the brood size for these worms was measured.

From each worm 10 L4 worms were transferred to 3cm plates individually. These worms were transferred to a fresh plate daily. After four days, the offspring were counted from each plate and the data evaluated with one-way ANOVA.

It was found that these two worms were still producing more offspring .These mutants worms were called *lat-1(ok1465)* mutant-1and *lat-1(ok1465)* mutant-2 (figure 3.6).

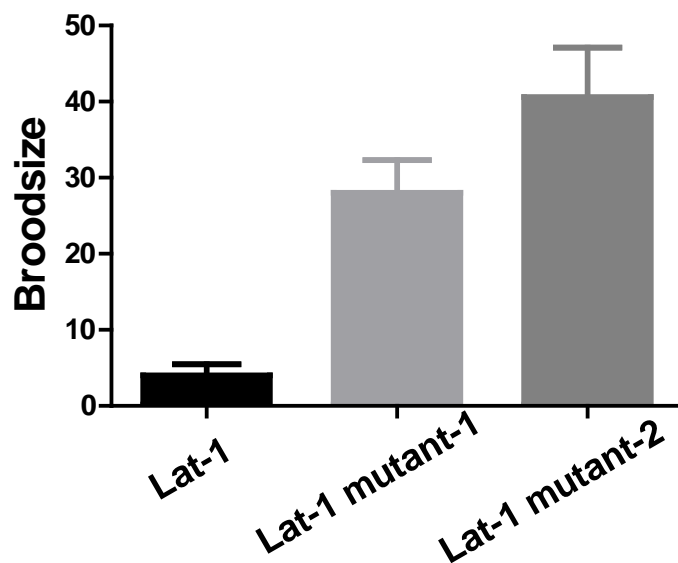


Figure 3.6 Testing the two mutant worms

The brood size of *lat-1*, *lat-1(ok1465)* mutant-1 and *lat-1(ok1465)* mutant-2 were counted. Ten L4 worm from every strain were transferred individually. After four days, the number of offspring was counted to get the main and standard deviation for every strain. P values were calculated relative to *lat-1(ok1465)* worms by one-way ANOVA analysis to determine level of significance.

It can be seen from graph 3.8 that the brood size for *lat-1(ok1465)* is 4 ± 4 . The brood size for *lat-1(ok1465)* mutant-1 is 28 ± 13 , whereas the brood size of *lat-1(ok1465)* mutant-2 is 40 ± 20 . There was a significant difference ($P < 0.05$) between *lat-1(ok1465)* brood size and *lat-1(ok1465)* mutant-1 brood size. In addition, there was a significant different ($P < 0.05$) between *lat-1(ok1465)* brood size and *lat-1(ok1465)* mutant-2 brood size. It was found that there is no significant difference between and *lat-1(ok1465)* mutant-1 brood size and *lat-1(ok1465)* mutant-2 brood size. Because of this and because they were from the same plate, they are probably the same mutant.

Section 3.6 Polymerase chain reaction (PCR)

A primer was designed to establish the presence of the *lat-1(ok1465)* deletion. This primer is located between 18185bp and 20395bp. the 3' primer binds at 20410-20432bp and the 5' primer binds at 18011-18030bp. Therefore, this primer just binds outside the deletion of *lat-1(ok1465)* worms. Another primer for *lat-1(ok1465)* in wild-type *C.elegans* was designed to amplify the region of the sequence deleted in the *lat-1(ok1465)* worms. The 3' primer binds at 19001-19020bp and the 5' primer binds at 18701-18720. This experiment was preformed to make sure the specificity of these primers.

Initially, a PCR reaction was set up with wild-type primers and wild-type genomic DNA, which was loaded onto lane 2 of the gel. The negative control was wild-type primers with *lat-1(ok1465)* (ok1465) genomic DNA which was loaded onto lane 3 of the gel. Then, a PCR reaction was set up with *lat-1(ok1465)* primers and *lat-1(ok1465)* genomic DNA, which was loaded onto lanes 4 of the gel. The negative control was *lat-1(ok1465)* primers with wild-type genomic DNA which was loaded onto lanes 5 of the gel. Figure 3.7 confirms the specificity of these primers.

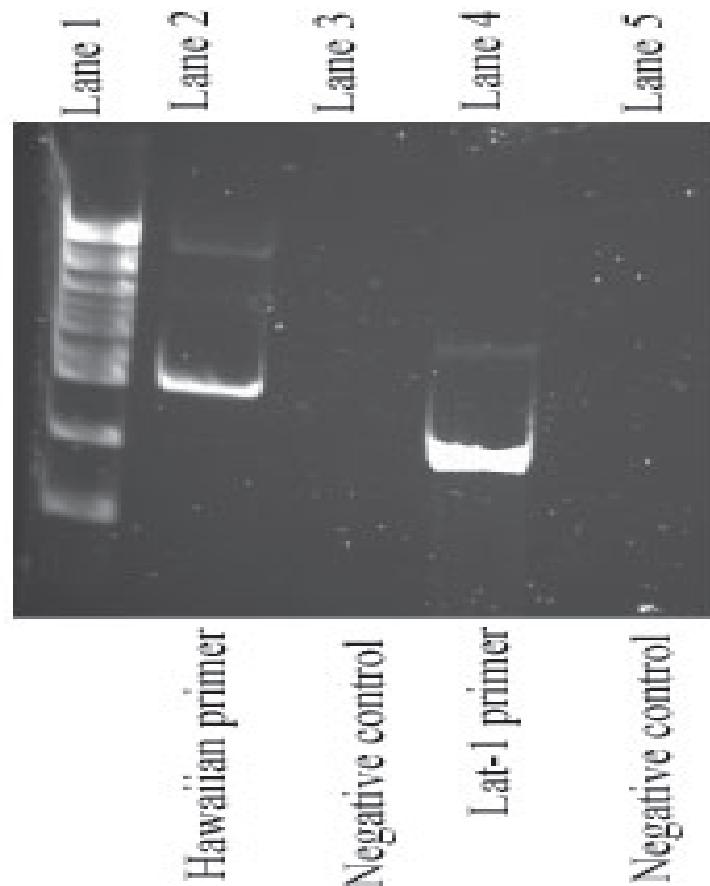


Figure 3.7 Acrylamide gel showing the specificity of *lat-1(ok1465)* gene primers

The *lat-1(ok1465)* primers were designed to establish the presence of this *lat-1(ok1465)* deletion. 30ul PCR reaction for *lat-1(ok1465)* primers and wild-type primer were set up (primers, wild-type genomic DNA or *lat-1(ok1465)* genomic DNA, and master mix). This was run for 35 cycles; 94°C for 30 seconds, 54°C for 30 seconds, 72°C for 1 minute. 3μl of loading buffer was added to each of the DNA samples and 100bp DNA ladder were gently mixed and run alongside a positive and negative control, on 12% polyacrylamide gel electrophoresis at 100V for 1 hour. To visualize bands in the gel, UV trans-illuminator was used and photographed by Bio Rad Gel-Doc system and the Quantity One software. Lane 1 has 100bp DNA ladder. Lane 2 has CB4856 *C.elegans* DNA with wild-type primers. L2 has OK1465 *C.elegans* DNA with wild-type primers, a negative control for lane 2. Lane 3 has *lat-1(ok1465)* *C.elegans* DNA with Lat1 primers. Lane 4 has CB4856 *C.elegans* DNA with Lat1 primers as a negative control for lane 3.

It can be seen from graph 3.7 that the length of primer of *lat-1(ok1465)* deletion is 214bp long as shown in lane 4. The length of *lat-1(ok1465)* primer of wild-type *C.elegans* is 320bp as shown in lane 2. No band was visible in Lane5 for the reaction *lat-1(ok1465)* primer with wild-type DNA. This is to confirm that the specificity of the *lat-1(ok1465)* primers only amplify DNA in a *lat-1(ok1465)* genome. In addition, no band was visible in Lane3 for the reaction wild-type primer with *lat-1(ok1465)* DNA. This is to confirm that the specificity of the wild-type primers only amplify DNA in a wild-type genome.

4. Discussion

Section 4.1 Liquid culture

At first experiments were performed using agar plates to grow large populations of worms but we found that it is time consuming to grow millions of worms on plates. According to Johnson & Wood, (1982) liquid media can be used instead of plates because the major problem associated with NGM plates is the life-cycle experiments that can be avoided by using liquid media (Johnson& Wood, 1982). So we decided to use liquid culture instead of agar plates. Different concentrations of bacteria (optical density) were used to define the best conditions for growth. Then, different amounts of liquid culture were used to determine the best amount of liquid culture in a one liter flask. Finally, the number of worms that can be grown in a particular amount of liquid culture was determined.

We found that increasing the concentration of bacteria in S medium might result in less adults and decline in growth curve. Because of this, the best bacterial concentration was measured. More than 1.7 O.D. at 600nm can reduce the yield of worms and will also kill the worms, presumably because of the lack of oxygen, as described in section 3.4.1. After that, we found that 400ml of liquid culture can be used in one liter flask. Our result show that the *lat-1(ok1465)* worms strain can be grown in liquid culture to produce sufficient numbers of adults at 20 °C to generate millions of nematodes within two weeks.

Growing *lat-1(ok1465)* in liquid culture takes longer than N2 worms, because of the low *lat-1(ok1465)* brood size. When a small number of *lat-1(ok1465)* worms were used, all the flasks had a large number of worms after nine days; in contrast, because of bacterial density some of the plates had good growth, some of them had small number of worms and no

worms in other plates. In addition, liquid cultures can be prepared more quickly than NGM agar plates. These results show that large numbers of worms can be grown in liquid culture, but that it is important to optimize this method so as to get reliable results.

Section 4.2 The *lat-1(ok1465)* mutation.

The gene knockout strategy is often very helpful for analysing gene function in *C. Elegans* (Strange, 2006). Therefore, latrophilin (*lat-1*) gene knockout worms and mutagenesis methods are required to study this issue. Self fertilization helps to study recessive mutations across the entire genome (Brenner, 1974). The *lat-1(ok1465)* worms have a deletion of *lat-1(ok1465)* gene conferred by *ok1465* allele as described in Section 1.2.4. It is known that most of the offspring of *lat-1(ok1465)* animals die during development, and the average brood size of *lat-1(ok1465)* worms is only four adults. The worms either arrest during embryogenesis or there is a failure of the gut to link up with the pharynx (Adenle, 2008). Therefore, *lat-1(ok1465)* mutants with an increase in brood size may be helpful to explain how the absence of *lat-1(ok1465)* causes a failure in development.

Section 4.3 Mutagenesis of *lat-1(ok1465)* worms

Mutagenesis can be used for genome-wide identification of gene knockouts. The mutagenesis strategy was performed to confirm that it works with *lat-1(ok1465)* worms and to create mutations with 50mM ethyl methanesulfonate (EMS). EMS is a common chemical mutagen for *C.elegans* (Brenner, 1974). To increase the probability of generating mutations that are derived from independent events, we used L4 animals as mutagenesis targets (Anderson., 1995). Moreover, if the effect of a toxin can be seen in the worms, resistance genes for that toxin can be identified using this method. Such a mutagenesis strategy might be helpful in identifying genes that are responsible for specific mechanisms of action (Dent,

2000). We hypothesized that random mutagenesis for the entire genome of *lat-1(ok1465)* worms might identify genes that are responsible for rescuing the lethality in the offspring of *lat-1(ok1465)* worms offspring. Subsequently, the location of this mutation can be determined and its function can be defined.

Section 4.4 Difficulty of dealing with *lat-1*

Section 4.4.1 Screening of *lat-1(ok1465)* worms

In order to mutate every gene in *C.elegans*, we need to treat a large number of animals with EMS as described above. The possibility of finding a mutation in a specific gene can be enhanced by screening a large number of mutagenized gametes or haploid genomes (Anderson, 1995).

The problem with the small brood size of *lat-1(ok1465)* worms is that screening for homozygous worms in the second generation might take a long time. In addition, to screen sufficient genomes in *lat-1(ok1465)* worms we need a large number of worms to be screened to get the specific genes, because the *lat-1(ok1465)* worms cannot produce large number of offspring since of them die before they reach adulthood. On the other hand, the benefit of the *lat-1(ok1465)* phenotype is that their small brood size phenotype makes the screening system more sensitive to detect an increase in brood size in a mutant that might provide valuable information about the mechanism of latrophilin signalling in *C.elegans*.

It was thought that screening the F2 *lat-1(ok1465)* worms would be difficult, because of the large number of *lat-1(ok1465)* worms that should be screened. This problem was mitigated after it was calculated that the screening strategy based on screening 20 *lat-1(ok1465)* worms in each plate would make it easy to see the difference between the brood

from 20 *lat-1(ok1465)* worms and the number of offspring when a mutant was present (described in section 3.5.2). Screening 20 worms per plate enables us to screen enough worms. As a practical consideration, it took approximately 8 hours to plate out 10000 worms during a screen. The stage of screening worm numbers took approximately 10 hours for 500 plates. Therefore, screening with 20 worms per plates allows for examination of approximately 10,000 worms in each mutagenesis process. We used this method to screen 10,000 worms within three weeks. It was found that screening 20 worms per plate is practical enough to do and so we can use 10000 worms in every mutagenesis screen and perform this experiment several times within two months.

Section 4.4.2 Culturing *lat-1(ok1465)* worms for mutagenesis

The *lat-1(ok1465)* worms were cultured for mutagenesis in two different ways. Firstly, *lat-1(ok1465)* worms were mutagenized and the P0 generation worms and the F1 generation worms were bleached to obtain a synchronized culture. Secondly, *lat-1(ok1465)* worms were left in the plate without any bleaching.

The brood size of *lat-1(ok1465)* worms is very low, when it was compared with wild type brood size. We found that the lethality in *lat-1(ok1465)* worms and the bleaching of P0 and F1 generate very low number of offspring. Although the bleaching process is good, the eggs which we got from some of the hermaphrodites do not hatch or die during developmental stages. Another disadvantage of bleaching is that it takes a longer time and more resources to produce a large number of *lat-1(ok1465)* worms which yields only a small number of worms. Because of the bleaching process, we have to start with many worms, which involve 20 liters of liquid culture, and practically this is very difficult to deal with, so we decided to culture the plates without bleaching.

Once we had enough worms they were divided into ten groups on 10 different plates. These worms were washed down from one generation to another rather than bleached between generations as described in section 2.2.6.2.2. There are many advantages of using this method. A much smaller number of *lat-1(ok1465)* worms was required for this experiment.

In spite of screening *lat-1(ok1465)* worms several times using the first method, we were not able to find any mutant with increased brood size. Two millions P0 worms were used for the first method with bleaching between generations. At the end of the 2nd generation we had only 2000 worms. When these 2000 worms were screened, the probability of getting a mutation in the specific gene was small (Brenner, 1974). In contrast, when 10,000 worms were used in the second method, we were able to screen 10,000 worms in the F2 generation. When these 10000 worms were screened, we found two mutant worms in one of the 10 groups.

The reason for dividing the population it into ten groups is to allow for the possible involvement of upto ten different genes. If two or more resistant worms were found in one group, they might have the same mutation. Dividing these worms into ten groups was found to be helpful, since it is easier to identify worms with different mutations. From this experiment, we found that the second method is better than the first method (bleaching).

Section 4.5 Screening plates for *lat-1(ok1465)* mutants with an increased brood size

A genetic screen was carried out for mutations that can increase the brood size in *lat-1(ok1465)* worms and to prevent the lethality in offspring caused by deletion in the *lat-1(ok1465)* signaling pathway in *C. elegans*. The *lat-1(ok1465)* worms were mutagenised with

50mM EMS and mutants were isolated that have increased brood size as described in section 3.5.4.

These results demonstrate that resistance to lethality in the offspring of *lat-1(ok1465)* worms can be induced by mutagenesis. These observations raise the possibility of getting stronger resistance to offspring lethality in *lat-1(ok1465)* worms. There could be couple of reasons for not getting more resistance genes. Although 800 plates were generated and screened, many plates have less than 20 worms. Therefore, it is possible sufficient genomes were not screened. Certainly, we have not screened every gene. If the 800 plates had exactly 20 worms in each, there will be 16000 worms which corresponds to 8000 F1 genomes (Brenner, 1974). 8000 genomes should be screened a couple of times to increase the probability of finding multiple resistance genes for *lat-1(ok1465)* lethality.

Section 4.5.1 Characterization of *lat-1(ok1465)* mutants with an increased brood size

There is clearly a different phenotype between *lat-1(ok1465)* mutant -1/-2 worms and *lat-1(ok1465)* worms, insofar as the brood size between *lat-1(ok1465)* mutant worms and *lat-1(ok1465)* worms is significantly different. However, the *lat-1(ok1465)* mutant -1/-2 worms exhibited no other obvious phenotype (data not shown). The *lat-1(ok1465)* worms are known to show an extended defecation cycle, and it would be of interest to determine if defecation cycle is reduced in these animals. The *lat-1(ok1465)* worms are known to die either during embryogenesis or during larval stage; so it would be interesting to find out if the reduced lethality is due to one or other of these stages being specifically affected.

Section 4.7 A genetic mapping system in *Caenorhabditis elegans*.

The reason for using genome mapping is to generate a high resolution map of a mutant gene in the genome. Thus, genes are identified and their functions can be investigated (Swan. K.A, 2002).

Mapping with single nucleotide polymorphisms has provided a significant benefit in the speed of positional cloning for many *C.elegans* researchers and has been developed (Jakubowski & Kornfeld 1999). Positional cloning of mutations used SNPs as genetic markers. SNPs do not possess a phenotype, unlike conventional marker mutations which were employed in traditional methods. Conventional marker mutations can mask some of the mutant phenotypes such as the behavioural defects that can be mapped using SNPs. It is possible to focus the site of mutation to area region of less than a single cosmid and finally to a single gene using SNP mapping (worm book).

It is known that CB4856 worms have an even distribution of SNPs across the chromosomes (Koch et al. 2000; Which *et al.*2001). CB4856 worms were isolated from a Hawaiian island and have a uniformly high density of polymorphisms compared with N2. These worms are nevertheless closely related when compared with Bristol N2 strain. It is estimated that genetic differences (the density of SNPs) between CB4856 and N2 occur on average every 1000 base pairs (bp), including substitutions, insertions or deletions. Although most polymorphisms between CB4856 and N2 are indeed single-nucleotide, small deletions and insertions are also common. Substitutions are more common than insertions and deletions. Substitutions are approximately 57% (3996 SNPs) transitions, but there are 44% (2996 SNPs) transversions. Polymorphisms are identified by RFLP analysis (snip-SNP).

Mutant worm were created by using EMS mutagenesis and resistant mutants are identified by screening. The resistant mutant in N2 worms is then bred with CB4856 males to generate heterozygous cross progeny which can be selfed to yield homozygous resistant mutant worms (worm book).

lat-1(ok1465) worms, on an N2 background have been bred with the CB4856 strain for six generation to generate a homozygous *lat-1(ok1465)* in the CB4856 background. EMS mutagenesis has been carried out to identify homozygous *lat-1(ok1465)* resistant mutant worms that show increased brood size. These mutants (ie m/m genotype) will be bred with F6 *lat-1(ok1465)* males on a CB4856 background. After that, the first generation would be selfed to get second generation, in which we should get 50 % +/+ (4 offspring), 25% m/+ (4 offspring) and 25 % m/m (~40 offspring). The m/m individuals will be selfed and DNA prepared from the F3 generation. After that, PCR will be set up using genomic DNA and primers designed to amplify the DNA regions containing the SNP. After amplification, the PCR products will be digested with a restriction enzyme specific to the SNP. Finally, these digested reactions will be loaded and run on a gel to identify N2 DNA that is associated with greater brood size in *lat-1(ok1465)* worms.

5. Conclusion and Future work

Our work shows that mutagenesis of *lat-1(ok1465)* worms was successful, and the screening procedure yielded a *lat-1(ok1465)* mutant with increased brood size, although we were not able to find mutants that produce 300 offspring just like wild type animals. In future, the mutagenesis for *lat-1(ok1465)* worms will be repeated, because sufficient genomes were not screened during this work. This might yield further genes which can further increase the brood size of *lat-1* animals. After that, we would perform mapping and identify the role of these mutant genes that confer increased brood size to *lat-1(ok1465)* worms, with the aim of understanding how *lat-1(ok1465)* signalling is involved in embryonic and larval growth.

6.Reference

- Adenle, A. (2008): Genetic analysis of latrophilin in the toxicity of combined latrotoxin for *C.elegans*. *School of Biology, Nottingham, University of Nottingham*. PhD: 173.
- Albert, P. S., and Riddle, D. L. (1988): Mutants of *Caenorhabditis-Elegans* That Form Dauer-Like Larvae. *Developmental Biology* **126**, 270-293.
- Ambros, V. (2000): Current Opinion in Genetics & Development **10**, 428-433.
- Anderson, P., 1995: Mutagenesis. *Methods Cell Biol.* **48**: 31-58.
- Antoshechkin I and Han M (1998): Five genes involved in *c.elegans* vulva development *West Coast Worm Meeting*.
- Bucher EA and Seydoux GC (1994): " Gastrulation in the nematode *Caenorhabditis elegans*." *Seminars in Developmental Biology* **5**:121-1
- BBC. (1998, 10 Dec). Small worm makes history. BBC [on-line]. Available: http://news.bbc.co.uk/hi/english/sci/tech/newsid_232000/232608.stm [1999, Jun 9].
- Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics* **77**:71-94.
- Cassada, R. C., and Russell, R. L. (1975): Dauerlarva, a Post-Embryonic Developmental Variant of Nematode *Caenorhabditis-Elegans*. *Developmental Biology* **46**, 326-342.

Chang G.W., Stacey.M., Kwakkenbos M.J., Hamann J., Gordon S. and H.H. Lin (2003):

Proteolytic cleavage of the EMR2 receptor requires both the extracellular stalk and the GPS motif, *FEBS Lett.* **547**, pp. 145–150.

Chang, M., Bellaoui, M., Boone, C. & Brown, G. W (2002): A genome-wide screen for

methyl methanesulfonate-sensitive mutants reveals genes required for S phase progression in the presence of DNA damage. *Proc. Natl Acad. Sci. USA* **99**, 16934–16939.

Cuppen.E.,E.Gort,et al.(2007).” Efficient target-selected mutagenesis in *Caenorhabditis elegans*: toward a knockout for every gene.” *Genome Res* **17**(5):649-58

Davletov, B.A., et al.(1996): Isolation and biochemical characterization of a Ca²⁺-independent alpha-latrotoxin-binding protein. *J Biol Chem*, **271**(38): p. 23239-45.#

De Bono M, Tobin DM, Davis MW, Avery L, Bargmann CI (2002): Social feeding in *Caenorhabditis elegans* is induced by neurons that detect aversive stimuli. *Nature* **419**:899–903

Dent JA, Smith MM, Vassilatis DK, Avery L (2000): The genetics of ivermectin resistance in *Caenorhabditis elegans*. *Proc Natl Acad Sci U S A* **97**: 2674–2679.

Donald, D. L. (Ed.). (1997): *C. elegans* II. New York, NY: Cold Spring Harbor Laboratory Press.

- Donkin S.G. and Dusenbery D.B. (1994): Using the *Caenorhabditis elegans* soil toxicity test to identify factors affecting toxicity of four metal ions in intact soil. *Water Air Soil Pollut*, **78**, 359-373.
- Finkelstein A, Rubin LL, Tzeng MC (1976): Black widow spider venom: effect of purified toxin on lipid bilayer membranes. *Science* **193**:1009–1011.
- Fraser, A. G. *et al.*(2000): Functional genomic analysis of *C. elegans* chromosome I by systematic RNA interference. *Nature* **408**, 325-330.
- Harmar A.J.,(2001): Family-B G-protein-coupled receptors, *Genome Biol.* **2** , pp. 3013.1–3013.10
- Hayflick J.S (2000): A family of heptahelical receptors with adhesion-like domains: a marriage between two super families, *J. Recept. Signal Transduct. Res.* **20** , pp. 119–131
- Hitchcock, D.; Black, M.; and Williams, P. (1997): Investigation into Using the Nematode *Caenorhabditis elegans* for Municipal and Industrial Wastewater Toxicity Testing. *Archives of Environmental Contamination and Toxicology* **33**:252-260.
- Hodgkin,j. and T.M. Barnes. (1991): More is not better: Brood size and population growth in a self-fertilizing nematode. *Proc. R. Soc. Lond. B Biol. Sci.* **246**: 19-24.
- Hodgkin, J., Horvitz, H., R. and Brenner, S. (1979): Nondisjunction mutants of the nematode *Caenorhabditis elegans*. *Genetics* **91**, 67-94.

Horvitz, H. R. (1997): A nematode as a model organism: the genetics of programmed_death [Film]. Cogito Learning Media, Inc. Available: <http://www.cogitomedia.com> [1999, Jul 20].

Ichtenko .K., Khvotchev M., Kiyatkin N., Simpson L., Sugita S. and Sudhof T.C., (1998): α -Latrotoxin action probed with recombinant toxin: receptors recruit α -latrotoxin but do not transduce an exocytotic signal, *EMBO J.* **17**, pp. 6188–6199.

Ichtenko K., Bittner M.A., Krasnoperov V., Little A.R., Chepurny O., Holz R.W. and Petrenko A.G.,(1999): A novel ubiquitously expressed α -latrotoxin receptor is a member of the CIRL family of G-protein-coupled receptors, *J. Biol. Chem.* **274**, pp. 5491–5498.

Jakubowski, J. and Kornfeld, K. (1999): A local, high-density, single-nucleotide polymorphism map used to clone

Johnson TE, Wood WB (1982): Genetic analysis of life span in *Caenorhabditis elegans*. *Proc Natl Acad Sci USA* **79(21)**:6603-6607.

Johnsen, R.C. and Baillie, D.L. (1991): Genetic analysis of a major segment [LGV (left)] of the genome of *Caenorhabditis elegans*. *Genetics* **129**: 735-752.

Kamath , R.S. and Ahringer, J. (2003): Genome-wide RNAi screening in *Caenorhabditis elegans*, *Methods* **30**, pp. 313–321.

- Khvotchev, M., G. Lonart, and Sudhof, T.C. (2000): *Role of calcium in neurotransmitter release evoked by alpha-latrotoxin or hypertonic sucrose*. *Neuroscience*, **101**(3):p. 793-802. (5)
- Kirill, E: et al (2004): Latrophilin fragments behave as independent proteins that associate and signal on binding of LTX, P. 4423–4433
- Kramer, J.M. (1997): Extracellular Matrix. In *C. elegans II* (ed. D.L. Riddle et al.) pp. 471-500. *Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York*
- Krasnoperov V.G., M.A. Bittner, W. Mo, L. Buryanovsky, T.A. Neubert, R.W. Holz, K. Ichtchenko and A.G. Petrenko, (2002): Protein tyrosine phosphatase- σ is a novel member of the functional family of α -latrotoxin receptors, *J. Biol. Chem.* **277**, pp. 35887–35895
- Krasnoperov, V.G., et al.,(1997): alpha-Latrotoxin stimulates exocytosis by the interaction with a neuronal G-protein-coupled receptor. *Neuron*,**18**(6): p. 925-37.
- Koch, R., van Luenen, H.G.A.M., van der Horst, M., Thijssen, K.L., and Plasterk, R.H.A. (2000): Single nucleotide polymorphisms in wild isolates of *Caenorhabditis elegans*. *Genome Res.* **10**: 1690–1696
- Liao, V.H. and Yu, C.W.(2001): *Caenorhabditis elegans* gcs-1 confers resistance to arsenic-induced oxidative stress, *BioMetals* 18 (2005), pp. 519–528 sistance, *Curr. Biol.* **11**, pp. 864–868

- Loechler, E.L., Green, C.L., and Essigmann, J.M. (1984): In vivo mutagenesis by O6-methylguanine built into a unique site in a viral genome. *Proc. Natl. Acad. Sci.* **81**: 6271–6275. EMS
- Loria P.M., J. Hodgkin and O. Hobert, (2004): A conserved postsynaptic transmembrane protein affecting neuromuscular signaling in *Caenorhabditis elegans*, *J. Neurosci.* **24**, pp. 2191–2201.
- Petrenko AG, Perin MS, Davletov BA, Ushkaryov YA, Geppert M, Südhof TC (1991): Binding of synaptotagmin to the alphasyntaxin receptor implicates both in synaptic vesicle exocytosis. *Nature* **353**:65–68
- Matsushita H., V.G. Leliana and Y.A. Ushkaryov, (1999): The latrophilin family: multiply spliced G protein-coupled receptors with differential tissue distribution, *FEBS Lett.* **443**, pp. 348–352.
- Mee, C. (2002). Molecular Analysis of the latrophilin receptor. Latrophilin in the nematode *Caenorhabditis elegans*. Nottingham, University of Nottingham.
- Mee, C. (2004). Molecular Analysis of the latrophilin receptor. Latrophilin in the nematode *Caenorhabditis elegans*. Nottingham, University of Nottingham.
- Meneely.P.,and Herman,R.(1979). Lethals, steriles and deficiencies in a region of the X chromosome of *C. elegans*. *Genetics* **92**:99-115.

- Al-anizy, M. (2006). Studies on Cytochrome P450 4X1. *Nottingham, University of Nottingham*.
- Nonet, M.L., et al., (1993): Synaptic function is impaired but not eliminated in *C. elegans* mutants lacking synaptotagmin. *Cell*, **73**(7): p. 1291-305.
- Rankin C.H. (2002). From gene to identified neuron to behaviour in *C. Elegans*. *Natural Review Genetics* **3**(8): 622–630
- Riddle, D., T. Blumenthal, B. Meyer, J. Priess (1997). *C. elegans* II. *New York, Cold Spring Harbor Laboratory Press*.
- Richmond, J., (2005): Synaptic function. *WormBook*,: p. 1-14.
- Schwarz, E.M., et al., (2006): *WormBase: better software, richer content*. *Nucleic Acids Res*, **34**(Database issue): p. D475-8.
- Smith, V., Botstein, D. & Brown, P.O. (1995): Genetic footprinting: a genomic strategy for determining a gene's function given its sequence. *Proc. Natl. Acad. Sci. USA* **92**, 6479–6483.
- Snow, E.T., Foote, R.S., and Mitra, S. (1984): Base-pairing properties of O6-methylguanine in template DNA during in vitro DNA replication. *J. Biol. Chem.* **259**: 8095–8100.

- Snyder D.A. A.M. Rivers, H. Yokoe, B.P. Menco and R.R. Anholt, (1991): Olfactomedin: purification, characterization, and localization of a novel olfactory glycoprotein, *Biochemistry* **30**, pp. 9143–9153
- Sudhof TC. (2001): α -Latrotoxin and its receptors: neuroligins and CIRL/latrophilins. *Annu Rev Neurosci.* **24**:933–962.
- Stacey M. H.H. Lin, S. Gordon and A.J. McKnight, (2000): LNB-TM7, a group of seven-transmembrane proteins related to family-B G-protein-coupled receptors, *Trends Biochem*
- Strange, K. (2006) “ *C.elegans* methods and applications” *Humana press*, NJ, USA.
- Sugita, S., et al., (1998): α -Latrotoxin receptor CIRL/latrophilin 1 (CL1) defines an unusual family of ubiquitous G-protein-linked receptors. G-protein coupling not required for triggering exocytosis. *J Biol Chem*,
- Sulston, J.E., (1983): Neuronal cell lineages in the nematode *Caenorhabditis elegans*. Cold Spring Harb Symp Quant Biol., **48** Pt 2: p. 443-52.
- Swan. K.A, C. D. E., McKusick. K.B, Voinov.A.V, Mapa.F.A., Cancilla.M.R. (2002). "High Throughput Gene Mapping in *Caenorhabditis elegans*." *Genome Research*: 1100-1105.
- Ushkaryov, Y. A., A. Rohou, A. Sugita et al. (2008): “ α -Latrotoxin and its receptors.” *Handb Exp Pharmacol* (**184**):171-206.

Volynski K.E., J.-P. Silva, V.G. Lelianova, M.A. Rahman, C. Hopkins and Y.A. Ushkaryov (2004):, Latrophilin fragments behave as independent proteins that associate and signal on binding of LTX^{N4C}, *EMBO J.* **23**, pp. 4423–4433.

W. L. Nicholas (1984): The Biology of Free-living Nematodes, *Clarendon, Oxford*

White J. (1988): The anatomy. In: The Nematode *Caenorhabditis elegans*, edited by Wood WB and The Community of *C. elegans* Researchers. *Cold Spring Harbor, NY: Cold Spring Harbor Laboratory*, p. 81–122.

Wicks, S.R., Yeh, R.T., Gish, W.R., Waterston, R.H., and Plasterk, R.H.A. (2001). Rapid gene mapping in *Caenorhabditis elegans* using a high density polymorphism map. *Nat. Genet.* **28**: 160–164.

Guest, M., et. al. (2007): "The calcium-activated potassium channel, SLO-1, is required for the action of the novel cyclo-octadepsipeptide anthelmintic, emodepside, in *Caenorhabditis elegans*." *The international journal for parasitology*, **37**: 1577-1588.

Willson, J., et al., (2004): Latrotoxin receptor signaling engages the UNC-13-dependent vesicle-priming pathway in *C. elegans*. *Curr Biol.* **14**(15): p. 1374-9.

Williams, P. and Dusenbery, D. (1988): Using the Nematode, *Caenorhabditis elegans*, to Predict Mammalian Acute Lethality to Metallic Salts. *Toxicology and Industrial Health* **4**(4):469-478.

Williams, P. and Dusenbery, D. (1990): Aquatic Toxicity Testing Using the Nematode, *Caenorhabditis elegans*. *Environmental Toxicology and Chemistry*. **9**(10):1285-1290.

Wood, W.B., (1988): Determination of pattern and fate in early embryos of *Caenorhabditis elegans*. *Dev Biol* (N Y 1985),**5**: p. 57-78.